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Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms

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This manual describes short-term (four- to eight-day) methods for estimating the chronic toxicity of effluents and receiving waters to a freshwater fish, an invertebrate, and an alga. Also included are guidelines on laboratory safety, quality assurance, facilities and equipment, dilution water, effluent sampling and holding, data analysis, and organism culturing and handling. Listings of computer programs for Dunnett's Procedure and Probit Analysis are provided in the Appendix.

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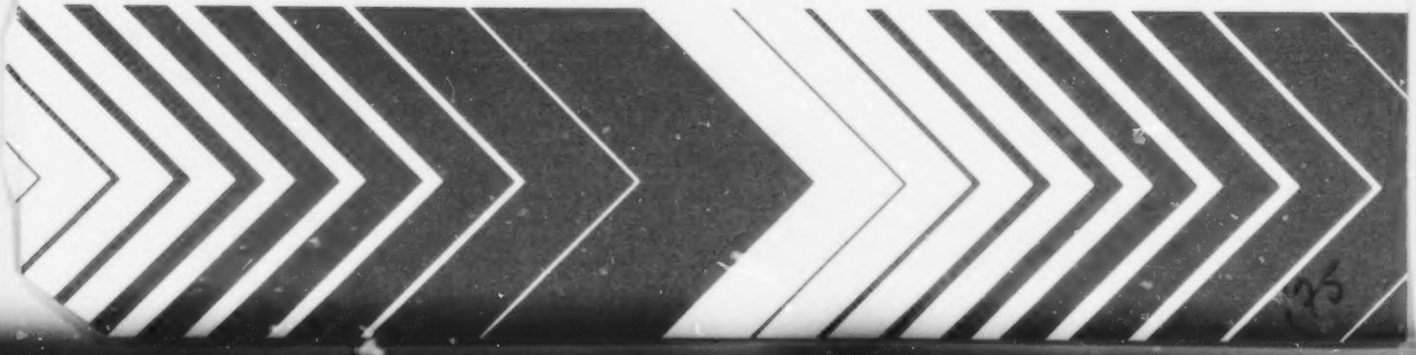

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SHORT-TERM METHODS FOR ESTIMATING THE CHRONIC TOXICITY OF EFFLUENTS AND RECEIVING WATERS TO FRESHWATER ORGANISMS

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NOTICE

This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

FOREWORD

Environmental measurements are required to determine the quality of ambient water, the character of effluents, and the effects of pollutants on aquatic life. The Environmental Monitoring and Support Laboratory - Cincinnati (EMSL-Cincinnati) operates an Agency-wide quality assurance program to assure standardization and quality control of systems for monitoring water and wastewater, and conducts research to develop, evaluate, and promulgate methods to:

- Measure the presence and concentration of physical, chemical, and radiological pollutants in water, wastewater, bottom sediments, and solid waste.
- Concentrate, recover, and identify enteric viruses, bacteria, and other microorganisms in water, wastewater, and municipal sludge.
- Measure the effects of pollution on freshwater, estuarine, and marine organisms, including the phytoplankton, zooplankton, periphyton, macrophyton, macroinvertebrates, and fish.
- Automate the measurement of the physical, chemical, and biological quality of water.

The Federal Water Pollution Control Act Amendments of 1972 (PL 92-500) and the Clean Water Act (CWA) of 1977 (PL 95-217) explicitly state that it is the national policy that the discharge of toxic substances in toxic amounts be prohibited. Determination of the toxicity of effluents, therefore, plays an important role in identifying and controlling toxic discharges to surface waters. This report provides standardized methods for estimating the chronic toxicity of effluents and receiving waters to aquatic life for use by the U.S. Environmental Protection Agency (USEPA) regional and state programs, and National Pollutant Discharge Elimination System (NPDES) permittees.

Robert L. Booth
Director
Environmental Monitoring and
Support Laboratory - Cincinnati

PREFACE

This manual is the first Agency methods manual for estimating the chronic toxicity of effluents and receiving waters. The draft was reviewed by the Bioassay Subcommittee of the EMSL-Cincinnati Biological Advisory Committee, USEPA headquarters and regional staff, other Federal agencies, state and interstate water pollution control programs, environmental protection groups, trade associations, major industries, consulting firms, academic institutions engaged in aquatic toxicology research, and other interested parties in the private sector.

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ABSTRACT

This manual describes short-term (four- to eight-day) methods for estimating the chronic toxicity of effluents and receiving waters to a freshwater fish, an invertebrate, and an alga. Also included are guidelines on laboratory safety, quality assurance, facilities and equipment, dilution water, effluent sampling and holding, data analysis, report preparation, and organism culturing and handling. Listings of computer programs for Dunnett's Procedure and Probit Analysis are provided in the Appendix.

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SECTION 1

INTRODUCTION

1.1 The Federal Water Pollution Control Act Amendments of 1972 (PL 92-500) and the Clean Water Act (CWA) of 1977 (PL 95-217) were enacted to restore and maintain the chemical, physical, and biological integrity of the Nation's waters (Section 101[a]), and contained specific or implied requirements for the collection of biomonitoring data in at least 15 sections.

1.2 The Declaration of Goals and Policy, Section 101(a)(3), in these two laws, states that "it is the national goal that the discharge of toxic pollutants in toxic amounts be prohibited." To achieve the goals of this legislation, extensive effluent toxicity screening programs were conducted during the 1970s by the regions and states. Acute toxicity tests (USEPA, 1975; Peltier, 1978) were used to measure effluent toxicity and to estimate the safe concentration of toxic effluents in receiving waters. However, for those effluents that were not sufficiently toxic to cause mortality in acute (one- to four-day) tests, short-term inexpensive methods were not available to detect the more subtle, low-level, long-term, adverse effects of effluents on aquatic organisms, such as reduction in growth and reproduction, and occurrence of terata. Fortunately, rapid developments in toxicity test methodology during the past five years have resulted in the availability of several methods that permit detection of the low-level, adverse effects (chronic toxicity) of effluents in eight days or less.

1.3 As a result of the increased awareness of the value of effluent toxicity test data for toxics control in the water quality program and the National Pollutant Discharge Elimination System (NPDES) permit program, which emerged from the extensive effluent toxicity monitoring activities of the regions and states, and the recent availability of short-term chronic toxicity test methods, the U. S. Environmental Protection Agency (USEPA) issued a national policy statement entitled, "Policy for the Development of Water Quality-Based Permit Limitations for Toxic Pollutants," in the Federal Register Vol. 49, No. 48, Friday, March 9, 1984. A technical support document on the use of effluent and receiving water toxicity data also has been prepared by the Office of Water Enforcement and Permits (OWEP) and the Office of Water Regulations and Standards (OWRS) to provide additional guidance on the implementation of the biomonitoring policy (USEPA, 1985).

1.4 This new Agency policy proposes the use of toxicity data to assess and control the discharge of toxic substances to the Nation's waters through the NPDES permits program. The policy states that "biological testing of effluents is an important aspect of the water quality-based approach for controlling toxic pollutants. Effluent toxicity data, in conjunction with other data, can be used to establish control priorities, assess compliance with State water quality standards, and set permit limitations to achieve those standards." All states have water quality standards which include narrative statements prohibiting the discharge of toxic materials in toxic amounts.

1.5 The four short-term tests described in this manual are for use in the NPDES Program to estimate one or more of the following: (1) the chronic toxicity of effluents collected at the end of the discharge pipe and tested with a standard dilution water (moderately hard synthetic freshwater in Table 1, p. 18); (2) the chronic toxicity of effluents collected at the end of the discharge pipe and tested with dilution water consisting of non-toxic receiving water collected upstream from the outfall, or with other uncontaminated surface water or standard dilution water having approximately the same hardness as the receiving water; (3) the toxicity of receiving water downstream from the outfall; and (4) the effects of multiple discharges on the quality of the receiving water. The tests may also be useful in developing site-specific water quality criteria.

1.6 These methods were developed to provide the most favorable cost-benefit relationship possible, and are intended for use in effluent toxicity tests performed on-site, where time is very costly, and for toxicity tests with effluent samples shipped to central and distant laboratories, where the volume of waste shipped must be kept to a minimum.

The tests include:

1. Seven-day, sub-chronic, fathead minnow (Pimephales promelas), static renewal, larval survival and growth test.

2. Seven-day, (three-brood), chronic, Ceriodaphnia dubia, static renewal, survival and reproduction test.

3. Eight-day, sub-chronic, fathead minnow (Pimephales promelas), static renewal, embryo-larval survival and teratogenicity test.

4. Four-day, chronic, Selenastrum capricornutum, static, growth test.

1.7 The first two tests were adapted from methods developed by Dr. Donald Mount and Teresa Norberg, Environmental Research Laboratory, USEPA, Duluth, Minnesota (Mount and Norberg, 1984; Norberg and Mount, 1985). The third test was adapted from a method developed by Drs. Wesley Birge and Jeffrey Black, Graduate Center for Toxicology, University of Kentucky, Lexington, Kentucky (Birge and Black, 1981). The fourth test, a 96-h, multi-generation test utilizing the freshwater alga, Selenastrum capricornutum, was adapted from the publications of the Environmental Research Laboratory - Corvallis (USEPA, 1971; Miller et al., 1978).

1.8 The Environmental Monitoring and Support Laboratory - Cincinnati (EMSL-Cincinnati) has incorporated the short-term chronic and sub-chronic tests into this manual for use by regulatory agencies involved in biological monitoring of wastewater under the NPDES program. Authority for promulgating test procedures for the analysis of pollutants is contained in Section 304(h) of the CWA.

1.9 The manual was prepared in the established EMSL-Cincinnati format (Kopp, 1983) so that each method can be used independently of the other methods.

SECTION 2

SHORT-TERM METHODS FOR ESTIMATING CHRONIC TOXICITY

2.1 The objective of aquatic toxicity tests with effluents or pure compounds is to estimate the "safe" or "no effect" concentration of these substances, which is defined as the concentration which will permit normal propagation of fish and other aquatic life in the receiving waters. The endpoints that have been considered in tests to determine the adverse effects of toxicants include death and survival, decreased reproduction and growth, locomotor activity, gill ventilation rate, heart rate, blood chemistry, histopathology, enzyme activity, olfactory function, and terata. Since it is not feasible to detect and/or measure all of these (and other possible) effects of toxic substances on a routine basis, observations in toxicity tests generally have been limited to only a few effects, such as mortality, growth, and reproduction.

2.2 Acute mortality is an obvious and easily observed effect, which accounts for its wide use in the early period of evaluation of the toxicity of pure compounds and complex effluents. The results of these tests were usually expressed as the concentration lethal to 50% of the test organisms (LC50) over relatively short exposure periods (two to four days).

2.3 As exposure periods of acute tests were lengthened, the LC50 and lethal threshold concentration were observed to decline for many compounds. By lengthening the tests to include one or more complete life cycles and observing the more subtle effects of the toxicants, such as a reduction in growth and reproduction, more accurate, direct, estimates of the threshold or safe concentration of the toxicant could be obtained. However, laboratory life-cycle tests may not accurately estimate the safe concentration of toxicants because they are conducted with a limited number of species under highly controlled, steady-state conditions, and the results do not include the effects of the stresses to which the organisms would ordinarily be exposed in the natural environment.

2.4 An early published account of a full life-cycle, fish toxicity test was that of Mount and Stephan (1967). In this study, fathead minnows (Pimephales promelas) were exposed to a graded series of pesticide concentrations throughout their life cycle, and the effects of the toxicant on survival, growth, and reproduction were measured and evaluated. This work was soon followed by full life-cycle tests using other toxicants and fish species.

2.5 McKim (1977) evaluated the data from 56 full life-cycle tests, 32 of which used the fathead minnow, and concluded that the embryo-larval and early juvenile life-stages were the most sensitive stages. He proposed the use of partial life-cyle toxicity tests with the early life-stages (ELS) of fish to establish water quality criteria.

2.6 Macek and Sleight (1977) found that exposure of critical life-stages of fish to toxicants provides estimates of chronically safe concentrations remarkably similar to those derived from full life-cycle toxicity tests, and reported that "for a great majority of toxicants, the concentration which will not be acutely toxic to the most sensitive life stages is the chronically safe concentration for fish, and that the most sensitive life stages are the embryos and fry." Critical life-stage exposure was considered to be exposure of the embryos during most, preferably all, of the embryogenic (incubation) period, and exposure of the fry for 30 days post-hatch for warm water fish with embryogenic periods ranging from one to 14 days, and for 60 days post-hatch for fish with longer embryogenic periods. They concluded that in the majority of cases, the maximum acceptable toxicant concentration (MATC) could be estimated from the results of exposure of the embryos during incubation, and the larvae for 30 days post-hatch.

2.7 Because of the high cost of full life-cycle fish toxicity tests and the emerging concensus that the ELS test data would be adequate for estimating chronically safe concentrations, there was a rapid shift by aquatic toxicologists to 30- to 90-day ELS toxicity tests for estimating chronically safe concentrations in the late 1970s. In 1980, USEPA adopted the policy that ELS test data could be used in establishing water quality criteria if data from full life-cycle tests were not available (USEPA, 1980a).

2.8 Published reports of the results of ELS tests indicate that the relative sensitivity of growth and survival as endpoints may be species dependent, toxicant dependent, or both. Ward and Parrish (1980) examined the literature on ELS tests that used embryos and juveniles of the salt water species, sheepshead minnow (Cyprinodon variegatus), and found that growth was not a statistically sensitive indicator of toxicity in 16 of 18 tests. They suggested that the ELS tests be shortened to 14 days posthatch and that growth be eliminated as an indicator of toxic effects.

2.9 In a review of the literature on 173 fish full life-cycle and ELS tests performed to determine the chronically safe concentrations of a wide variety of toxicants, such as metals, pesticides, organics, inorganics, detergents, and complex effluents, Woltering (1984) found that at the lowest effect concentration, significant reductions were observed in fry survival in 57%, fry growth in 36%, and egg hatchability in 19% of the tests. He also found that fry survival and growth were very often equally sensitive, and concluded that the growth response could be deleted from routine application of the ELS tests. The net result would be a significant reduction in the duration and cost of screening tests with no appreciable impact on estimating MATCs for chemical hazard assessments. Benoit, et al., (1982), however, in early life-stage tests with four organic chemicals, found larval growth to be the most significant measure of effect, and survival to be equally or less sensitive than growth.

2.10 Efforts to further reduce the length of partial life-cycle toxicity tests for fish without compromising their predictive value have resulted in the development of an eight-day, embryo-larval survival and teratogenicity test for fish and and other aquatic vertebrates (Birge and Black, 1981; Birge et al., 1985), and a seven-day larval survival and growth test (Mount et al., 1984; Norberg and Mount, 1985).

2.11 The similarity of estimates of chronically safe concentrations of toxicants derived from short-term, embryo-larval survival and teratogenicity test to those derived from full life-cycle tests has been demonstrated by Birge et al. (1981), Birge and Cassidy (1983), and Birge et al. (1985).

2.12 Use of a seven-day, fathead minnow larval survival and growth test was first proposed by Norberg and Mount at the 1983 annual meeting of the Society for Environmental Toxicology and Chemistry (Norberg and Mount, 1983). This test was subquently used by Mount and associates in field demonstrations at Lima, Ohio (Mount, et al., 1984), and at many other locations. Growth was frequently found to be more sensitive than survival in determining the effect of complex effluents.

2.13 Norberg and Mount (1985) performed three single toxicant fathead minnow larval growth tests with zinc, copper, and DURSBAN^{R}, using dilution water from Lake Superior. The results were comparable to, and had confidence intervals that overlapped with, chronic values reported in the literature for both ELS and full life-cycle tests.

2.14 Efforts to develop a short-term cladoceran chronic toxicity test as a substitute for the 21- to 28-day *Daphnia* chronic toxicity test have resulted in a seven-day (three-brood) survival and reproduction test using *Ceriodaphnia* (Mount and Norberg, 1984). This test has been shown to provide data comparable to the previous, longer-term *Daphnia* chronic tests, and has also been used extensively by Mount and associates in field demonstrations.

2.15 The algal toxicity test described in this manual is a shortened version of the Agency's algal growth potential test (Miller et al., 1978). The 96-h length of the toxicity test period spans several generation times and, therefore, more than meets the requirements for a full life-cycle chronic test.

2.16 The use of short-term, subchronic and chronic toxicity tests in the NPDES Program is especially attractive because they provide a more direct estimate of the safe concentration of effluents in receiving waters than was provided by acute toxicity tests, at an only slightly increased level of effort, compared to that required by the fish full life-cyle chronic and (30-day) ELS tests and the 21- to 28-day cladoceran tests.

SECTION 3

HEALTH AND SAFETY[1]

3.1 GENERAL PRECAUTIONS

3.1.1 Collection and use of effluents in toxicity tests may involve significant risks to personal safety and health. Personnel collecting effluent samples and conducting toxicity tests should take all safety precautions necessary for the prevention of bodily injury and illness which might result from ingestion or invasion of infectious agents, inhalation or absorption of corrosive or toxic substances through skin contact, and asphyxiation due to lack of oxygen or presence of noxious gases.

3.1.2 Prior to sample collection and laboratory work, personnel should determine that all necessary safety equipment and materials have been obtained and are in good condition.

3.2 SAFETY EQUIPMENT

3.2.1 Personal Safety Gear

Personnel should use safety equipment, as required, such as rubber aprons, laboratory coats, respirators, gloves, safety glasses, hard hats, and safety shoes.

3.2.2 Laboratory Safety Equipment

Each laboratory (including mobile laboratories) should be provided with safety equipment such as first aid kits, fire extinguishers, fire blankets, emergency showers, and eye fountains.

3.3 GENERAL LABORATORY AND FIELD OPERATIONS

3.3.1. Work with effluents should be performed in compliance with accepted rules pertaining to the handling of hazardous materials (see safety manuals, Paragraph 3.5). It is recommended that personnel collecting samples and performing toxicity tests should not work alone.

3.3.2. Because the chemical composition of effluents is usually only poorly known, they should be considered as potential health hazards, and exposure to them should be minimized.

3.3.3. It is advisable to cleanse exposed parts of the body immediately after collecting effluent samples.

3.3.4. All containers should be adequately labeled to indicate their contents.

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[1] Adapted from: Peltier and Weber (1985).

3.3.5. Good housekeeping contributes to safety and reliable results.

3.3.6. Electrical equipment or extension cords not bearing the approval of Underwriter Laboratories must not be used. Ground-fault interrupters must be installed in all "wet" laboratories where electrical equipment is used.

3.3.7. Mobile laboratories should be properly grounded to protect against electrical shock.

3.4 DISEASE PREVENTION

3.4.1 Personnel handling samples which are known or suspected to contain human wastes should be immunized against tetanus, typhoid fever, and polio.

3.5 SAFETY MANUALS

3.5.1 For further guidance on safe practices when collecting effluent samples and conducting toxicity tests, check with the permittee and consult general industrial safety manuals, including USEPA (1977), and Walters and Jameson (1984).

SECTION 4

QUALITY ASSURANCE[1]

4.1 INTRODUCTION

4.1.1 Quality Assurance (QA) practices for effluent toxicity tests consist of all aspects of the test that affect data quality, such as: (1) effluent sampling and handling; (2) the source and condition of the test organisms; (3) condition of equipment; (4) test conditions; (5) instrument calibration; (6) replication; (7) use of reference toxicants; (8) record keeping; and (9) data evaluation. For general guidance on good laboratory practices related to toxicity testing, see: FDA, 1978; USEPA, 1979d, 1980b, and 1980c; and DeWoskin, 1984.

4.2 EFFLUENT AND RECEIVING WATER SAMPLING AND HANDLING

4.2.1 Effluent samples collected for on-site and off-site testing must be preserved as described in Section 8, Effluent and Receiving Water Sampling and Sample Handling.

4.3 TEST ORGANISMS

4.3.1 The test organisms used in the procedures described in this manual are the fathead minnow, Pimephales promelas, the cladoceran, Ceriodaphnia dubia, and the green alga, Selenastrum capricornutum. The organisms used should be disease-free, and should be positively identified to species.

4.4 FACILITIES, EQUIPMENT, AND TEST CHAMBERS

4.4.1 Laboratory and bioassay temperature control equipment must be adequate to maintain recommended test water temperatures. Recommended materials must be used in the fabrication of the test equipment which comes in contact with the effluent (see Section 5, Facilities and Equipment).

4.5 ANALYTICAL METHODS

4.5.1 Routine chemical and physical analyses must include established quality assurance practices outlined in Agency methods manuals (USEPA, 1979a,b).

4.6 CALIBRATION AND STANDARDIZATION

4.6.1 Instruments used for routine measurements of chemical and physical parameters such as pH, DO, temperature, conductivity, alkalinity, and hardness, must be calibrated and standardized according to instrument manufacturers procedures as indicated in the general section on quality assurance (see EPA Methods 150.1, 360.1, 170.1, and 120.1, USEPA, 1979b).

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[1] Adapted from: Peltier (1978), Peltier and Weber (1985), and USEPA (1979a).

4.6.2 Wet chemical methods used to measure hardness and alkalinity must be standardized according to the procedures for those specific EPA methods (see EPA Methods 130.2 and 310.1, USEPA 1979b).

4.7 DILUTION WATER

4.7.1 The dilution water used in the toxicity tests may be synthetic water, receiving water, or ground water, appropriate to the objectives of the study and logistical constraints, as discussed in Section 7.

4.8 TEST CONDITIONS

4.8.1 Water temperature must be maintained within the limits specified for each test. Dissolved oxygen (DO) concentrations and pH should be checked at the beginning of the test and daily throughout the test period.

4.9 TEST ACCEPTABILITY

4.9.1 The results of the fathead minnow or *Ceriodaphnia* 24-h reference toxicant tests are unacceptable if the survival in the controls is less than 90%. For effluent toxicity tests to be acceptable, control survival must be at least 80%. The results of the algal toxicity test are unacceptable if the cell density in the controls after 96 h is less than 10^6 cells/mL.

4.9.2 An individual test may be conditionally acceptable if temperature, DO, and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the tests (see test condition summaries). The acceptability of the test would depend on the best professional judgment and experience of the investigator. The deviation from test specifications must be noted when reporting data from the test.

4.10 PRECISION

4.10.1 The ability of the laboratory personnel to obtain consistent, precise results must be demonstrated with reference toxicants before they attempt to measure effluent toxicity. The single laboratory precision of each type of test to be used in a laboratory should be determined by performing five or more tests with a reference toxicant. In cases where the test data are used in the Probit Analysis (see Section 9), precision can be described by the mean, standard deviation, and relative standard deviation (percent coefficient of variation, or CV) of the calculated end points from the replicated tests. However, in cases where the results are reported in terms of the No-Observed-Effect Concentration (NOEC) and Lowest-Observed-Effect Concentration (LOEC) (see Section 9), precision can only be described by listing the NOEC-LOEC interval for each test. In this case, it is not possible to express precision in terms of a commonly used statistic. For instance, when all tests of the same toxicant yield the same NOEC-LOEC interval, maximum precision has been attained. However, the "true" no effect concentration could fall anywhere within the interval, NOEC ± (NOEC-LOEC).

4.10.2 It should be noted here that the dilution factor selected for a test determines the width of the NOEC-LOEC interval and the inherent maximum precision of the test. As the absolute value of the dilution factor decreases, the width of the NOEC-LOEC interval increases, and the inherent maximum precision of the test decreases. When a dilution factor of 0.3 is used, the NOEC could be considered to have a relative variability as high as ± 300%. With a dilution factor of 0.5, the NOEC could be considered to have a relative variability of ± 100%. Other factors which can affect test precision include test organism age, condition, and sensitivity, temperature control, and feeding.

4.11 REPLICATION AND TEST SENSITIVITY

4.11.1 The sensitivity of the tests will depend in part on the number of replicates, the probability level selected, and the type of statistical analysis. The minimum recommended number of replicates varies with the test and the statistical method used, and is discussed in Section 9 and in each method. The sensitivity of the test will increase as the number of replicates is increased.

4.12 QUALITY OF TEST ORGANISMS

4.12.1 If the laboratory does not have an ongoing test organism culturing program and obtains the test organisms from an outside source, the sensitivity of each batch of test organisms must be evaluated with a reference toxicant in a toxicity test performed concurrently with the effluent and/or receiving water toxicity tests. If the laboratory maintains breeding cultures, the sensitivity of the offspring should be determined in a toxicity test performed with a reference toxicant at least once each month. If preferred, this reference toxicant test may be performed concurrently with an effluent toxicity test. A 24-h acute toxicity test is used to determine the sensitivity of fathead minnows and *Ceriodaphnia*. For the acute toxicity test design, see Peltier and Weber (1985). A 96-h toxicity test is used to determine the sensitivity of *Selenastrum*.

4.12.2 The sensitivity of fathead minnow larvae is determined with newly hatched larvae as used in the growth and survival or embryo-larval test. The sensitivity of *Ceriodaphnia* is determined using animals less than 24 h old, and which were released within the same 4-h period.

4.12.3 Three reference toxicants are available from EMSL-Cincinnati to establish the precision and validity of toxicity data generated by biomonitoring laboratories: sodium dodecylsulfate (SDS), sodium pentachlorophenate (NaPCP), and cadmium chloride ($CdCl_2$). The reference toxicants may be obtained by contacting the Quality Assurance Branch, Environmental Monitoring and Support Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio, 45268; FTS 684-7325, comm'l 513-569-7325. Instructions for the use and the expected toxicity values for the reference toxicants are provided with the samples. To assure comparability of QA data on a national scale, all laboratories must use the same source of reference toxicant (EMSL-Cincinnati) and the same formulation of dilution water -- moderately hard synthetic water, described in Table 1, p. 18, for fathead minnows and *Ceriodaphnia*, and algal growth medium described in Tables 1 and 2, Section 14, for *Selenastrum*.

4.13 FOOD QUALITY

4.13.1 The quality of the food for fish and invertebrates is an important factor in toxicity tests. Suitable trout chow, *Artemia*, and other foods must be obtained as described in the manual. Limited quantities of reference *Artemia* cysts, information on commerical sources of good quality *Artemia* cysts, and procedures for determining cyst suitability as food are available from the Quality Assurance Branch, Environmental Monitoring and Support Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio, 45268. The suitability of each new supply of food must be determined in a side-by-side test in which the response of test organisms fed with the new food is compared with the response of organisms fed a reference food or a previously used, satisfactory food.

4.14 CONTROL CHARTS

4.14.1 A control chart should be prepared for each reference-toxicant-organism combination, and successive toxicity values should be plotted and examined to determine if the results are within prescribed limits (Fig. 1). In this technique, a running plot is maintained for the toxicity values (X_i) from successive tests with a given reference toxicant. The type of control chart illustrated (USEPA, 1979a) is used to evaluate the cumulative trend of the statistics from a series of samples. The mean ($\bar{X}$) and upper and lower control limits ($\pm$ 2S) are re-calculated with each successive point, until the statistics stabilize. Outliers, which are values which fall outside the upper and lower control limits, and trends of increasing or decreasing sensitivity are readily identified. At the $P_{0.05}$ probability level, one in 20 tests would be expected to fall outside of the control limits by chance alone.

4.14.2 If the toxicity value from a given test with the reference toxicant does not fall in the expected range for the test organisms when using the standard dilution water, the sensitivity of the organisms and the overall credibility of the test system are suspect. In this case, the test procedure should be examined for defects and should be repeated with a different batch of test organisms.

4.15 RECORD KEEPING

4.15.1 Proper record keeping is required. Bound notebooks should be used to maintain detailed records of the test organisms such as species, source, age, date of receipt, and other pertinent information relating to their history and health, and information on the calibration of equipment and instruments, test conditions employed, and test results. Annotations should be made on a real-time basis to prevent the loss of information.

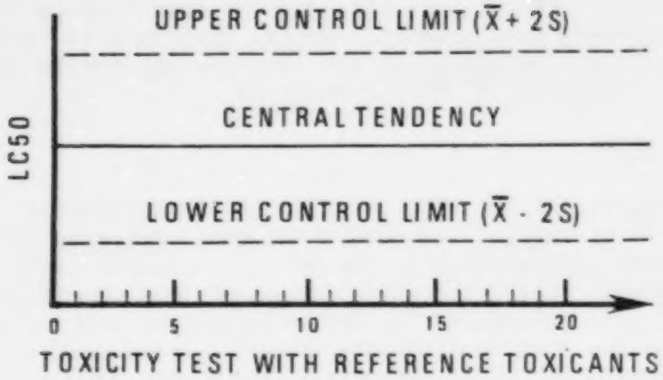
UPPER CONTROL LIMIT (X̄ + 2S)
CENTRAL TENDENCY
LOWER CONTROL LIMIT (X̄ - 2S)
LC50
0
5
10
15
20
TOXICITY TEST WITH REFERENCE TOXICANTS

Figure 1. Control chart.

$$\bar{X} = \frac{\sum_{i=1}^{n} X_i}{n}$$

$$S = \sqrt{\frac{\sum_{i=1}^{n} X_i^2 - \left(\sum_{i=1}^{n} X_i\right)^2 / n}{n - 1}}$$

Where:

X_i = Successive LC50's from toxicity tests.

n = Number of tests.

$\bar{X}$ = Mean LC50.

S = Standard deviation.

SECTION 5

FACILITIES AND EQUIPMENT[1]

5.1 GENERAL REQUIREMENTS

5.1.1 Effluent toxicity tests may be performed in a fixed or mobile laboratory. Facilities should include equipment for rearing, holding, and acclimating organisms. Temperature control can be achieved using circulating water baths, heat exchangers, or environmental chambers. Water used for rearing, holding, acclimating, and testing organisms may be ground water, surface water, dechlorinated tap water, or synthetic water. Dechlorination can be accomplished by aeration (allowing the water to stand in an open vessel for 24 h), carbon filtration, or the use of sodium thiosulfate. Use of 1.0 mg (anhydrous) sodium thiosulfate/L will reduce 1.5 mg chlorine/L. After dechlorination, total residual chlorine should be non-detectable. Air used for aeration must be free of oil and fumes. Test facilities must be well ventilated and free of fumes. During rearing, holding, acclimating, and testing, test organisms should be shielded from external disturbances.

5.1.2 Materials used for exposure chambers, tubing, etc., which come in contact with the effluent should be carefully chosen. Tempered glass and perfluorocarbon plastics (TEFLON^R) should be used whenever possible to minimize sorption and leaching of toxic substances. These materials may be reused following decontamination. Plastics such as polyethylene, polypropylene, polyvinyl chloride, TYGON^R, etc., may be used as test chambers or to store effluents, but caution should be exercised in their use because they could introduce toxicants when new, or carry over toxicants from one test to another if reused. The use of glass carboys is discouraged for safety reasons.

5.1.3 New plastic products of a type not previously used should be tested for toxicity before initial use by exposing the test organisms in the test system where the material is used. Equipment (pumps, valves, etc.) which cannot be discarded after each use because of cost, must be decontaminated according to the cleaning procedures listed below. Fiberglass, in addition to the previously mentioned materials, can be used for holding, acclimating, and dilution water storage tanks, and in the water delivery system. All material should be flushed or rinsed thoroughly with the test media before using in the test. Copper, galvanized material, rubber, brass, and lead must not come in contact with holding, acclimation, or dilution water, or with effluent samples and test solutions. Some materials, such as several types of neoprene rubber (commonly used for stoppers) may be toxic and should be tested before use.

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[1] Adapted from: Peltier and Weber (1985).

5.1.4 Silicone adhesive used to construct glass test chambers absorbs some organochlorine and organophosphorus pesticides, which are difficult to remove. Therefore, as little of the adhesive as possible should be in contact with water. Extra beads of adhesive inside the containers should be removed.

5.2 TEST CHAMBERS

5.2.1 Test chamber size and shape are varied according to size of the test organism. Requirements are specified in each test.

5.3 CLEANING

5.3.1 New plasticware used for sample collection or organism exposure vessels does not require rigorous cleaning. It is sufficient to rinse the new containers once with sample before use. New glassware, however, should be soaked overnight in acid (see below).

5.3.2 It is recommended that all sample containers, test vessels, pumps, tanks, and other equipment that has come in contact with effluent be washed after use in the manner described below to remove surface contaminants. Special cleaning requirements for glassware used in algal toxicity tests are described in Section 14.

1. Soak 15 minutes, and scrub with detergent in tap water, or clean in an automatic dishwasher.
2. Rinse twice with tap water.
3. Carefully rinse once with fresh dilute (20% V:V) nitric acid or hydrochloric acid to remove scale, metals and bases. To prepare a 20% solution of acid, add 20 mL of concentrated acid to 80 mL of distilled water.
4. Rinse twice with tap water.
5. Rinse once with full-strength acetone to remove organic compounds.
6. Rinse well with tap water.
7. Rince twice with dilution water.

5.3.3 All test chambers and equipment must be thoroughly rinsed with the dilution water immediately prior to use in each test.

SECTION 6

TEST ORGANISMS

6.1 SPECIES

6.1.1 The organisms used in the chronic tests described in this manual are the fathead minnow, (Pimephales promelas), the cladoceran, Ceriodaphnia dubia, and the green alga, Selenastrum capricornutum.

6.2 SOURCE

6.2.1 The test organisms are easily cultured in the laboratory. Culturing, care, and handling procedures for Ceriodaphnia and Selenastrum are described in the respective test methods sections. A fathead minnow culturing procedure using laboratory water is described in Peltier and Weber (1985).

6.2.2 Starter cultures of Selenastrum capricornutum are available from the following sources:

1. Aquatic Biology Section, Biological Methods Branch, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268.

2. Environmental Research Laboratory, U.S. Environmental Protection Agency, 200 SW 35th Street, Corvallis, Oregon 97330.

3. American Type Culture Collection (Culture No. ATCC 22662), 12301 Parklawn Drive, Rockville, Maryland 10852.

4. Culture Collection of Algae, Botany Department, University of Texas, Austin, Texas 78712.

6.2.3 Starter cultures of the fathead minnow (Pimephales promelas) and Ceriodaphnia dubia (Berner, 1985) can be obtained from the Aquatic Biology Section, Biological Methods Branch, EMSL-Cincinnati Newtown Facility, Environmental Monitoring and Support Laboratory, U. S. Environmental Protection Agency, Newtown, Ohio 45244 (Phone: FTS 778-8350; Commercial 513-527-8350).

6.2.4 If because of their source there is any uncertainty concerning the identity of the organisms, it is advisable to have them examined by a second party to confirm their identification.

6.3 SHIPMENT

6.3.1 Many states have strict regulations regarding the importation of non-native fishes. Required clearances should be obtained from state fisheries agencies before arrangements are made for the interstate shipment of fathead minnows.

6.4 DISPOSAL

6.4.1 Test organisms must be destroyed after use.

SECTION 7

DILUTION WATER

7.1 The source of dilution water used in the tests will depend largely on the objectives of the study as described in Section 1: (1) If the objective of the test is to estimate the inherent chronic toxicity of the effluent, a standard dilution water (moderately hard water, Table 1) is used; (2) If the objective of the test is to estimate the chronic toxicity of the effluent in uncontaminated receiving water, the test may be conducted using dilution water consisting of a single grab sample of non-toxic receiving water collected upstream from the outfall, or with other uncontaminated surface water or standard dilution water having approximately the same hardness (± 10%) as the receiving water; (3) If the objective of the test is to determine the additive effects of the discharge on already contaminated receiving water, the test is performed using dilution water consisting of receiving water collected daily upstream from the outfall.

7.2 When the dilution water is to be taken from the receiving water "upstream" from the outfall, it should be collected at a point as close as possible to the outfall, but upstream from or outside of the zone influenced by the effluent. The sample should be collected immediately prior to the test, but never more than 96 h before the test begins. Except where it is used within 24 h, the sample should be chilled to 4°C during or immediately following collection, and maintained at that temperature until used.

7.3 Where toxicity-free dilution water is required in a test, the water is considered acceptable if test organisms show adequate survival, growth, and reproduction in the controls during the test.

7.4 Dechlorinated water should be used as dilution water only as a last resort, because it is usually difficult to completely remove all the residual chlorine or chlorinated organics, which may be very toxic to the test organisms. Sodium thiosulfate is recommended for dechlorination (1.0 mg anhydrous sodium thiosulfate/L will reduce 1.5 mg chlorine/L). After dechlorination, total residual chlorine must be non-detectable.

7.5 If it is necessary to pass the dilution water through a deionizer to remove unacceptably high concentrations of copper, lead, zinc, fluoride, or other toxic substances before use, it must be reconstituted to restore the calcium and magnesium removed by the deionization process.

7.6 To prepare a synthetic fresh water, use the reagents listed in Table 1. For example, to prepare 20 L of moderately hard synthetic water:

1. Place 19 L of distilled or deionized water in a properly cleaned plastic carboy.
2. Add sufficient $MgSO_4$, $NaHCO_3$ and KCl to the carboy, and stir well.

3. Add sufficient $CaSO_4.2H_2O$ to 1 L of distilled or deionized water in a separate flask, place on a magnetic stirrer until the calcium sulfate has dissolved and add to the carboy and stir well.
4. Aerate vigorously for 24 h (with air filtered through cotton to remove oil) to dissolve the added chemicals and stabilize the medium.

7.7 The measured pH, hardness and alkalinity of the aerated water will be approximately as indicated under "Final Water Quality" in Table 1.

TABLE 1. PREPARATION OF SYNTHETIC FRESH WATER[a]

	Reagent Added (mg/L)[b]				Final Water Quality		
Water Type	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCL	pH[c]	Hardness[d]	Alkalinity[d]
Very soft	12.0	7.5	7.5	0.5	6.4-6.8	10-13	10-13
Soft	48.0	30.0	30.0	2.0	7.2-7.6	40-48	30-35
Moderately Hard	96.0	60.0	60.0	4.0	7.4-7.8	80-100	60-70
Hard	192.0	120.0	120.0	8.0	7.6-8.0	160-180	110-120
Very hard	384.0	240.0	240.0	16.0	8.0-8.4	280-320	225-245

[a] Taken in part from Marking and Dawson (1973).

[b] Add reagent grade chemicals to distilled or deionized water.

[c] Approximate equilibrium pH after 24 h of aeration.

[d] Expressed as mg $CaCO_3$/L .

SECTION 8

EFFLUENT AND RECEIVING WATER SAMPLING AND SAMPLE HANDLING

8.1 EFFLUENT SAMPLING

8.1.1 The effluent sampling point usually should be the same as that specified in the NPDES discharge permit (USEPA, 1979c). Conditions for exception would be: (1) better access to a sampling point between the final treatment and the discharge outfall; (2) if the processed waste is chlorinated prior to discharge to the receiving waters, it may also be desirable to take samples prior to contact with the chlorine to determine toxicity of the unchlorinated effluent; or (3) in the event there is a desire to evaluate the toxicity of the influent to municipal waste treatment plants or separate wastewater streams in industrial facilities prior to their being combined with other wastewater streams or non-contact cooling water, additional sampling points may be chosen.

8.1.2 The decision on whether to collect grab or composite samples is based on the objectives of the test and an understanding of the short and long-term operations and schedules of the discharger. If the effluent quality varies considerably with time, which can occur where holding times are short, grab samples may seem preferable because of the ease of collection and the potential of observing peaks (spikes) in toxicity. However, the sampling duration of a grab sample is so short that full characterization of an effluent over a 24-h period would require a prohibitive number of separate samples and tests. Collection of a 24-h composite sample, however, may dilute toxicity spikes, and averages the quality of the effluent over the sampling period. A lengthy discussion of the advantages and disadvantages of using grab or composite samples is found in Peltier and Weber (1985).

8.1.3 Aeration during collection and transfer of effluents should be minimized to reduce the loss of volatile chemicals.

8.1.4 Definitive tests performed for NPDES permit purposes require daily effluent sample collection and daily renewal of test solutions.

8.2 RECEIVING WATER SAMPLING

8.2.1 It is common practice to collect grab samples for receiving water toxicity studies.

8.2.2 When non-toxic receiving water is required for a test, it may be collected upstream from the outfall or from other uncontaminated surface water having approximately the same hardness (± 10%) as the receiving water. If the objective of the test is to determine the additive effects of the discharge on receiving water which may already be contaminated, the test is performed using dilution water consisting of receiving water collected daily upstream from the outfall.

8.2.3 Dilution water to be taken from the receiving water "upstream" from the outfall, is collected at a point as close as possible to the outfall, but upstream from or outside of the zone influenced by the effluent.

8.2.4 To determine the extent of the zone of toxicity in the receiving water downstream from the outfall, receiving water samples are collected at several distances downstream from the discharge. The time required for the effluent-receiving-water mixture to travel to sampling points downstream from the outfall may be difficult to ascertain, and it may not be possible to correlate downstream toxicity with effluent toxicity at the discharge point unless a dye study is performed. The toxicity of receiving water samples from five stations downstream from the discharge point can be evaluated using the same number of test vessels and test organisms as used in one effluent toxicity test with five effluent dilutions.

8.3 SAMPLE HANDLING AND PRESERVATION

8.3.1 If the data from the samples are to be acceptable for use in the NPDES Program, the lapsed time from collection of a grab or composite sample and the initiation of the test must not exceed 72 h. Composite samples should be chilled during collection, where possible. Except when used within 24 h of collection, samples must be chilled after collection and maintained at 4°C until used.

8.3.2 Samples Used in On-Site Tests

8.3.2.1 Samples collected for on-site tests should be used within 24 h.

8.3.3 Samples Shipped to Off-Site Facilities

8.3.3.1 Samples collected for off-site toxicity testing are to be chilled to 4°C when collected, shipped iced to the central laboratory, and there transferred to a refrigerator (4°C) until used. Every effort must be made to initiate the test with an effluent sample on the day of arrival in the laboratory.

8.3.3.2 Samples may be shipped in 4-L (1-gal) glass jugs, CUBITAINERS^R, or new plastic "milk" jugs. All sample containers should be rinsed with source water before being filled with sample. Glass jugs can be cleaned and reused (see p. 12), whereas CUBITAINERS^R and plastic jugs are not reused. Plastic containers used for effluents or toxic surface water samples should be punctured after use to prevent reuse.

8.4 SAMPLE PREPARATION

8.4.1 With the *Ceriodaphnia* and fathead minnow tests, effluents and surface waters must be filtered through a (30 um) plankton net to remove indigenous organims that may attack or be confused with the test organisms (see *Ceriodaphnia* test method for details). Surface waters used in algal toxicity tests must be filtered through a 0.45 um pore diameter filter before use. It may be necessary to first coarse-filter the dilution and/or waste water through a nylon sieve having 2- to 4-mm holes to remove debris and/or break up large floating or suspended solids.

8.4.2 The DO concentration in the dilution water should be near saturation prior to use. Aeration will bring the DO and other gases into equilibrium with air, minimize oxygen demand, and stabilize the pH.

8.4.3 If the dilution water and effluent must be warmed to bring them to the prescribed test temperature, supersaturation of the dissolved gases may become a problem. To prevent this problem, the effluent and dilution water are heated to 25°C and checked for dissolved oxygen (DO) with a probe. If the DO exceeds 8.5 mg/L (100% saturation), the solutions are aerated virorously with an air stone (usually 1-2 min) until the DO is lowered to 100% saturation.

SECTION 9

CHRONIC TOXICITY TEST END POINTS AND DATA ANALYSIS

9.1 END POINTS

9.1.1 Numerous terms are used to define the end points employed in chronic toxicity tests, which have their origin in the earlier, full life-cycle tests. As shorter "chronic" tests were developed, it became common practice to apply the same terminology to the end points. The primary terms in current use are listed below:

9.1.1.1 Safe Concentration - the highest concentration which will permit normal propagation of fish and other aquatic life in receiving waters.

9.1.1.2 No Observed Effect Concentration (NOEC) - The highest concentration of toxicant to which organisms are exposed in a full life-cycle or partial life-cycle test, which causes no statistically significant adverse effect on the observed parameters (usually hatchability, survival, growth, and reproduction).

9.1.1.2. Lowest Observed Effect Concentration (LOEC) - The lowest concentration of toxicant to which organisms are exposed in a life-cycle or partial life-cycle test, which causes a statistically significant adverse effect on the observed parameters (usually hatchability, survival, growth, and reproduction).

9.1.1.3. Maximum Acceptable Toxicant Concentration (MATC) - An undetermined concentration within the interval bounded by the NOEC and LOEC.

9.1.1.4. Chronic Value (ChV) - A value lying between the NOEC and LOEC; derived by calculating the geometric mean of the NOEC and LOEC. The term is sometimes used interchangably with MATC.

9.1.1.5. LC (or EC) - Lethal concentration (LC) or effective concentration (EC). A point estimate of the toxicant concentration that would adversely affect a given percent of the test organisms, calculated by regression (such as Probit Analysis). The LC1 (or EC1) is the estimated concentration of toxicant that adversely affects 1% of the test population, and is defined here as the threshold concentration, or lowest concentration that would cause an adverse effect on the observed parameters, and falls in the range of the NOEC and LOEC (Birge, et. al. 1981).

9.2 DATA ANALYSIS

9.2.1 Role of the Statistician

9.2.1.1 The choice of a statistical method to analyze toxicity test data and the interpretation of the results of the analysis of the data from any of the toxicity tests described in this manual can become problematic because of the inherent variability and sometimes unavoidable anomalies in biological data. Analysts who are not proficient in statistics are strongly advised to seek the assistance of a statistician before selecting the method of analysis and using any of the results.

9.2.2 Plotting the Data

9.2.2.1 It is recommended that the data always be plotted as a preliminary step, to help spot problems and detect unsuspected trends or patterns in the responses.

9.2.3 Data Transformations

9.2.3.1 Transformations of the data, such as arc sine and logs, can be used if they help the data meet the assumptions of the proposed analyses.

9.2.4 Analysis of Growth and Reproduction Data

9.2.4.1 Growth data from the fathead minnow larval survival and growth test, and reproduction data from the *Ceriodaphnia* survival and reproduction test, are analyzed using Dunnett's Procedure (Dunnett, 1955) if the assumptions of normality and homogeneity of variance are met (see Appendix for details). If the assumptions are not met, the data are analyzed using Steel's Many-One Rank Test (Steel, 1959; Miller, 1981).

9.2.4.2 The growth response data from the algal toxicity test may be converted to a proportion of the growth of the controls, which may then be analyzed by Probit Analysis (Finney, 1971) or, the growth response data, after an appropriate transformation if necessary to meet the assumptions of normality and homogeneity of variance, may be analyzed by Dunnett's Procedure or Steel's Many-One Rank Test.

9.2.5 Analysis of Mortality Data

9.2.5.1 Mortality data from the fathead minnow larval survival and growth test and the fathead minnow embryo-larval survival and teratogenicity test are used in a Probit Analysis to determine the LC1, if Probit Analysis is appropriate (see discussion below).

9.2.5.2 Fisher's Exact Test is used to analyze the mortality data from the *Ceriodaphnia* survival and reproduction test, prior to the analysis of the reproduction data.

9.2.5.3 Mortality data from the fathead minnow larval survival and growth test, and the fathead minnow embryo-larval survival and teratogenicity test, can be analyzed by Dunnett's Procedure or Steel's Many-One Rank Test after transforming the square root of the proportion of dead organisms to an arc sine value (see Appendix). This transformation is performed by the computer program for Dunnett's Procedure provided in the Appendix.

9.2.6 Dunnett's Procedure

9.2.6.1 Dunnett's Procedure consists of an analysis of variance (ANOVA) to determine the error term, which is then used in a multiple comparison method for comparing each of the treatment means with the control mean, in a series of paired tests. Use of Dunnett's Procedure requires at least two replicates per treatment.

9.2.6.2 The asumptions upon which the use of Dunnett's Procedure are contingent are that the observations are independent and normally distributed, with homogeneity of variance. Before analyzing the data, the assumptions are checked using the procedures provided in the Appendix.

9.2.6.3 Some indication of the sensitivity of the analysis should be provided by calculating: (1) the minimum difference between means that can be detected as statistically significant, and (2) the percent change from the control mean that this minimum difference represents for a given test. Calculation of beta levels (Type II error, which results when the null hypothesis is not rejected when it should be) as an indication of the power of the test would be another alternative.

9.2.6.4 The safe concentration derived from this test is reported in terms of the NOEC. A step-by-step example of Dunnett's Procedure is provided in the Appendix.

9.2.6.5 If, after suitable transformations have been carried out, the normality assumptions have not been met, the Steel Many-One Rank Test should be used.

9.2.7 Steel's Many-One Rank Test

9.2.7.1 Steel's Many-One Rank Test is a multiple comparison method for comparing several treatments with a control which is similar to Dunnett's Procedure, except that it is not necessary to meet the assumption for normality. The data are ranked, and the analysis is performed on the ranks rather than on the data themselves. If the data are normally or nearly normally distributed, Dunnett's Procedure would be more sensitive (would detect smaller differences between the treatments and control). For data that are not normally distributed, Steel's Many-One Rank Test can be much more efficient (Hodges and Lehmann, 1956). It is necessary to have at least four replicates to use Steel's test. The sensitivity of this test cannot be stated in terms of the minimum difference between treatment means and the control mean.

9.2.7.2 The safe concentration is reported as the NOEC. A step-by-step example of Steel's Many-One Rank Test is provided in the Appendix.

9.2.8 Probit Analysis

9.2.8.1 Probit Analysis is used to analyze percentage data from concentration-response tests. The analysis can provide an estimate of the concentration of toxicant lethal to a given percent of the test organisms and provide a confidence interval for the estimate. Probit Analysis also assumes normal distribution of log tolerances and independence of the individual responses. To use Probit Analysis, at least two partial mortalities must be obtained.

9.2.9 Fisher's Exact Test

9.2.9.1 Fisher's Exact Test (Finney, 1948; Pearson and Hartley, 1962) is a statistical method based on the hypergeometric probability distribution that can be used to test that the probability of a response is the same in two binomial populations. When used with the *Ceriodaphnia data*, it provides a conservative test of the equality of any two survival proportions assuming only the independence of responses from a binomial population.

SECTION 10

REPORT PREPARATION[1]

The following general format and content are recommended for the report:

10.1 INTRODUCTION

1. Permit number
2. Toxicity testing requirements of permit
3. Plant location
4. Name of receiving water body
5. Contractor (if contracted)
   a. Name of firm
   b. Phone number
   c. Address

10.2 PLANT OPERATIONS

1. Product(s)
2. Raw materials
3. Operating schedule
4. Description of waste treatment
5. Schematic of waste treatment
6. Retention time (if applicable)
7. Volume of waste flow (MGD, CFS, GPM)

10.3 SOURCE OF EFFLUENT (AMBIENT) AND DILUTION WATER

1. Effluent Samples
   a. Sampling point
   b. Collection dates and times
   c. Sample collection method
   d. Physical and chemical data

2. Surface Water Samples
   a. Sampling point
   b. Collection dates and times
   c. Sample collection method
   d. Physical and chemical data
   e. Streamflow (at 7Q10 and at time of sampling)

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[1] Adapted from: Peltier and Weber (1985). Prepared by Lee Tebo and William Peltier, Environmental Services Division, U. S. Environmental Protection Agency, Athens, Georgia.

3. Dilution Water Samples
    a. Source
    b. Collection date and time
    c. Pretreatment
    d. Physical and chemical characteristics

10.4 TEST METHODS

1. Toxicity test method used
2. End point(s) of test
3. Deviations from reference method, if any, and the reason(s)
4. Date and time test started
5. Date and time test terminated
6. Type of test chambers
7. Volume of solution used/chamber
8. Number of organisms/test chamber
9. Number of replicate test chambers/treatment
10. Acclimation of test organisms (mean and range)
11. Test temperature (mean and range)

10.5 TEST ORGANISMS

1. Scientific name
2. Age
3. Life stage
4. Mean length and weight (where applicable)
5. Source
6. Diseases and treatment (where applicable)

10.6 QUALITY ASSURANCE.

1. Standard toxicant used and source
2. Date and time of most recent test
3. Dilution water used in test
4. Results (LC50 or, where applicable, NOEC and/or EC1)
5. Physical and chemical methods used

10.7 RESULTS

1. Provide raw biological data in tabular form, including daily records of affected organisms in each concentration (including controls)
2. Provide table of LC50's, NOECs, etc
3. Indicate statistical methods to calculate end points
4. Provide summary table of physical and chemical data
5. Tabulate QA data

SECTION 11

TEST METHOD[1,2]

FATHEAD MINNOW (PIMEPHALES PROMELAS) LARVAL SURVIVAL AND GROWTH TEST METHOD 1000.0

1. SCOPE AND APPLICATION

1.1 This method estimates the chronic toxicity of whole effluents and receiving water to the fathead minnow (*Pimephales promelas*), using newly hatched larvae in a seven-day, static renewal test. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

1.2 Detection limits of the toxicity of an effluent or pure substance are organism dependent.

1.3 Single or multiple excursions in acute toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly volatile and highly degradeable toxicants in the source may not be detected in the test.

1.4 This method should be restricted to use by or under the supervision of professionals experienced in aquatic toxicity testing.

2. SUMMARY OF METHOD

2.1 Larvae (preferrably less than 24-h old) are exposed in a static renewal system for seven days to different concentrations of effluent or to receiving water. Test results are based on the survival and growth (increase in weight) of the larvae.

3. DEFINITIONS

(Reserved for addition of terms at a later date.)

4. INTERFERENCES

4.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities and Equipment).

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[1] The format used for this method was taken from Kopp, 1983.

[2] This method was adapted from Norberg and Mount, 1985.

4.2 Adverse effects of low dissolved oxygen (DO) concentrations, high concentrations of suspended and/or dissolved solids, and extremes of pH, may mask the presence of toxic substances.

4.3 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling and Sample Handling).

4.4 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival, and confound test results.

4.5 Food added during the test may sequester metals and other toxic substances and confound test results.

5. SAFETY

5.1 See Section 3, Health and Safety.

6. APPARATUS AND EQUIPMENT

6.1 Fathead minnow and brine shrimp culture units -- see Peltier and Weber (1985). This test requires 150-300 newly hatched larvae. It is preferable to obtain this fish from an inhouse fathead minnow culture unit. If it is not feasible to culture fish inhouse, newly hatched larvae can be shipped in well oxygenated water in insulated containers.

6.2 Samplers -- automatic sampler, preferrably with sample cooling capability, that can collect a 24-h composite sample of 4 L.

6.3 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling and Sample Handling).

6.4 Environmental chamber or equivalent facility with temperature control ($25 \pm 2^{o}C$).

6.5 Water purification system -- Millipore Super-Q or equivalent.

6.6 Balance -- analytical, capable of accurately weighing larvae to 0.0001 g.

6.7 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of the weighing pans and the expected weights of the pans plus fish.

6.8 Test chambers -- borosilicate glass or non-toxic disposable plastic labware. A minimum of two 1-L beakers are required for each concentration and control. To avoid potential contamination from the air, the chambers should be covered during the test.

6.9 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

6.10 Volumetric pipets-- Class A, 1-100 mL.

6.11 Serological pipets-- 1-10 mL, graduated.

6.12 Pipet bulbs and fillers -- Propipet^R, or equivalent.

6.13 Droppers, and glass tubing with fire polished edges, 4mm ID -- for transferring larvae.

6.14 Wash bottles -- for washing embryos from substrates and containers and for rinsing small glassware and instrument electrodes and probes.

6.15 Glass or electronic thermometers -- for measuring water temperatures.

6.16 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

6.17 National Bureau of Standards Certified thermometer (see USEPA Method 170.1, USEPA 1979b).

6.18 pH, DO, and specific conductivity meters -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of the above parameters, a portable, field-grade instrument is acceptable.

6.19 Miscellaneous apparatus and equipment -- transfer containers, pumps, and automatic dilution devices should be constructed of materials as indicated in Section 5, Facilities and Equipment.

7. REAGENTS AND CONSUMABLE MATERIALS

7.1 Reagent water -- defined as activated-carbon-filtered distilled or deionized water that does not contain substances which are toxic to the test organisms. A water purification system may be used to generate reagent water (see paragraph 6.5 above).

7.2 Effluent, surface water, and dilution water -- see Section 7, Dilution Water, and Section 8, Effluent and Surface Water Sampling and Sample Handling.

7.3 Reagents for hardness and alkalinity tests (see USEPA Methods 130.2 and 310.1, USEPA 1979b).

7.4 pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) for standards and calibration check (see USEPA Method 150.1, USEPA 1979b).

7.5 Membranes and filling solutions for dissolved oxygen probe (see USEPA Method 360.1, USEPA 1979b), or reagents for modified Winkler analysis.

7.6 Laboratory quality assurance samples and standards for the above methods.

7.7 Specific conductivity standards (see USEPA Method 120.1, USEPA 1979b).

7.8 Reference toxicant solutions (see Section 4, Quality Assurance).

7.9 Formalin (4%) for use as a preservative for the fish larvae.

7.10 Brine Shrimp (Artemia) Cysts -- see Peltier and Weber (1985).

7.10.1 Although there are many commercial sources of brine shrimp eggs, the Brazilian or Colombian strains are preferred because the supplies examined have had low concentrations of chemical residues. (One source is Aquarium Products, 180 L Penrod Ct., Glen Burnie, MD, 21061). Each new batch of Artemia cysts should be evaluated for nutritional suitability against known suitable reference cysts by performing a larval growth test. It is recommended that a sample of newly-hatched Artemia nauplii from each new batch of cysts be chemically analyzed to determine that the concentration of total organic chlorine does not exceed 0.15 ug/g wet weight or the total concentration of organochlorine pesticides plus PCBs does not exceed 0.3 ug/g wet weight (USEPA, 1982). If those values are exceeded, the Artemia should not be used.

7.10.2 Limited quantities of reference Artemia cysts, information on commerical sources of good quality Artemia cysts, and procedures for determining cyst suitability are available from the Quality Assurance Branch, Environmental Monitoring and Support Laboratory, U. S. Environmental Protection Agency, Cincinnati, Ohio, 45268.

7.11 Test organisms -- Newly-hatched fathead minnow larvae (see Peltier and Weber, 1985).

8. SAMPLE COLLECTION, PRESERVATION AND STORAGE

8.1 See Section 8, Effluent and Receiving Water Sampling and Sample Handling.

9. CALIBRATION AND STANDARDIZATION

9.1 See Section 4, Quality Assurance.

10. QUALITY CONTROL

10.1 See Section 4, Quality Assurance.

11. PROCEDURES

11.1 TEST SOLUTIONS

11.1.1 Surface Waters

11.1.1.1 Surface water toxicity is determined with samples used directly as collected.

11.1.2 Effluents

11.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. One of two dilution factors, approximately 0.3 or 0.5, is commonly used. A dilution factor of approximately 0.3 allows testing between 100% and 1% effluent using only five effluent concentrations (100%, 30%, 10%, 3%, and 1%). This series of dilutions minimizes the level of effort, but because of the wide interval between test concentrations provides poor test precision (± 300%). A dilution factor of 0.5 provides greater precision (± 100%), but requires several additional dilutions to span the same range of effluent concentrations. Improvements in precision decline rapidly as the dilution factor is increased beyond 0.5

11.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used, beginning at 10%. If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions at the lower range of effluent concentrations can be added.

11.1.2.3 The volume of effluent required for daily renewal of two replicates per concentration, each containing 500 mL of test solution, is approximately 2 L. Prepare enough test solution (approximately 1400 mL) at each effluent concentration to provide 400 mL additional volume for chemical analyses.

11.2 START OF THE TEST

11.2.1 Tests should begin as soon as possible, preferably within 24 h of sample collection. If the persistence of the sample toxicity is not known, the maximum holding time should not exceed 36 h. In no case should the test be started more than 72 h after sample collection. Just prior to testing, the temperature of the sample should be adjusted to (25 ± 2°C) and maintained at that temperature until portions are added to the dilution water.

11.2.2 The test is initiated by placing larvae one or two at a time, into each test chamber in sequential order, until each chamber contains 10 larvae, for a total of at least 20 larvae for each concentration. The test organisms should come from a pool of larvae consisting of at least three separate spawnings. The amount of water added to the chambers when transferring the larvae to the compartments should be kept to a minimum to avoid unnecessary dilution of the test concentrations.

11.2.3 Randomize the position of test chambers at the beginning of the test.

11.3 LIGHT, PHOTOPERIOD AND TEMPERATURE

11.3.1 The light quality and intensity should be at ambient laboratory levels, which is approximately 10-20 uE/m^2/s, or 50 to 100 foot candles (ft-c), with a photoperiod of 16 h of light and 8 h of darkness. The water temperature in the test chambers should be maintained at 25 ± 2°C.

11.4 DISSOLVED OXYGEN (DO)

11.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain satisfactory DO concentrations. The DO concentrations should not fall below 40% saturation. If it is necessary to aerate, all concentrations and the control should be aerated. The aeration rate should not exceed 100 bubbles/min, using a pipet with an orifice of approximately 1.5 mm, such as a 1-mL, Kimax serological pipet, No. 37033, or equivalent. Care should be taken to ensure that turbulence resulting from aeration does not cause undue physical stress to the fish.

11.5 FEEDING

11.5.1 The fish in each test chamber are fed 0.1 mL (approximately 700 to 1000) of a concentrated suspension of newly hatched (less than 24-h old) brine shrimp nauplii three times daily at 4-h intervals (at the beginning, midway, and at the end of the work day). The nauplii should be rinsed with freshwater before use.

11.6 DAILY CLEANING OF TEST CHAMBERS

11.6.1 Before the daily renewal of test solutions, uneaten and dead brine shrimp and other debris are removed from the bottom of the test chambers with a siphon hose. Alternately, a large pipet (50 mL) fitted with a rubber bulb can be used. Because of their small size during the first few days of the tests, larvae are easily drawn into the siphon tube when cleaning the test chambers. By placing the test chambers on a light box, inadvertent removal of larvae can be greatly reduced because they can be more easily seen. If the water siphoned from the test chambers is collected in a white plastic tray, the larvae caught up in the siphon can be retrieved and returned to the chambers.

11.7 TEST SOLUTION RENEWAL

11.7.1 The test solutions are renewed daily using freshly collected samples, immediately after cleaning the test chambers. The water level in each chamber is lowered to a depth of 7 to 10 mm, which leaves 15 to 20% of the test solution. New test solution (500 mL) should be added slowly by pouring down the side of the test chamber to avoid excessive turbulence for the larvae.

11.8 ROUTINE CHEMICAL AND PHYSICAL ANALYSIS

11.8.1 At a minimum, the following measurements are made:

11.8.1.1 DO is measured at the beginning and end of each 24-h exposure period at all test concentrations and in the control.

11.8.1.2 Temperature, pH, and conductivity are measured at the beginning of each 24-h exposure period at all test concentrations and in the control.

11.8.1.3 Alkalinity and hardness are measured at the beginning of each 24-h exposure period in 100% effluent and in the control.

11.9 OBSERVATIONS DURING THE TEST

11.9.1 The number of live and dead larvae in each test chamber are recorded daily, and the dead larvae are discarded.

11.9.2 Protect the larvae from unnecessary disturbance during the test by carrying out the daily test observations, solution renewals, and removal of dead larvae, carefully. Make sure the larvae remain immersed during the performance of the above operations.

11.10 TERMINATION OF THE TEST

11.10.1 The test is terminated after seven days of exposure. At termination, the larvae in each test chamber are counted and preserved as a group, in 4% formalin, and are dried and weighed at a later date. Immediately prior to the dry weight analysis, the preserved larvae are rinsed in distilled water. The group of rinsed larvae from each test chamber are transferred to a tared weighing boat and dried at 100°C for a minimum of 2 h. Immediately upon removal from the drying oven, the weighing boats are placed in a dessicator to prevent the absorption of moisture from the air, until weighed. The weights should be measured to the nearest 0.1 mg.

11.11 ACCEPTABILITY OF TEST RESULTS

11.11.1 For the test results to be acceptable, survival in the controls must be at least 80%, except where survival in any test concentration is 80% or better.

11.12 SUMMARY OF TEST CONDITIONS

11.12.1 A summary of test conditions is listed in Table 1.

12. CALCULATIONS

12.1 The endpoints of toxicity tests using the fathead minnow larvae are based on the adverse effects on survival and growth. Probit Analysis (Finney, 1971), Dunnett's Procedure (Dunnett, 1955), and Steel's Many-One Rank Test (Steel, 1959; Miller, 1981), are used to evaluate the data. See the Appendix for examples of the manual computations, and the program listings and examples of data input and program output.

12.2 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. Tests for normality and homogeneity of variance are included in the Appendix. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

12.3 Table 2 shows the survival and growth response after seven days of exposure to the reference toxicant NaPCP. The data for an effluent would be similar except that the test concentrations would be expressed as a percent.

12.4 Analysis of Survival Data - Probit Analysis

12.4.1 Probit Analysis (Finney, 1971) is used to determine the concentration causing 1% mortality (LC1). In this analysis, the total survival data from all test replicates at a given concentration are combined (see Table 2, Mean Proportion Surviving). A listing of the computer program and an example of data input and program output for the Probit Analysis are provided in the Appendix. Note that for the data in Table 2, the number of animals exposed in each concentration would be 40, and number that died at each concentration could be calculated from the proportions in the table. The program transforms the concentration values to $\log_{10}$ and the percent mortality to probits, and then performs a regression analysis.

12.4.2 Report the LC1 and its 95% confidence limits. The LC1 is an estimate of the threshold (chronically toxic) concentration. For the sample data in Table 2, the LC1 is 128 ug NaPCP/L, with upper and lower 95% confidence intervals of 30.5 ug/L and 198 ug/L, respectively.

12.4.3 If the data do not meet the assumptions necessary for the Probit analysis, analyze the data using Dunnett's Test, as described below.

12.5 Analysis of Survival Data - Dunnett's Procedure

12.5.1 The survival data meet the normality assumptions (see Appendix), which indicates that analysis by Dunnett's Procedure is appropriate. If the data had not met the assumptions, Steel's Many-One Rank Test would have been used (see Section 9). Dunnett's Procedure (Dunnett, 1955; Steel and Torrie, 1960) includes an analysis of variance (ANOVA), followed by a comparison of each toxicant concentration mean with the control mean. The error value calculated in the ANOVA is used in the comparison of the control and treatment means.

12.5.2 It is necessary to have at least duplicate test chambers at each treatment concentration to perform this test.

12.5.3 The computer program listed in the Appendix generates output which includes an ANOVA table, a statement about each treatment mean that can be used to identity the NOEC and LOEC, and the minimum difference between treatment and control means that can be detected as statistically significant.

12.5.4 The computer program makes the necessary transformation of the survival data by converting the square root of the proportion of surviving organisms to arc sine during the analysis, and includes a special modification of the arc sine transformation which is required where the proportion of surviving organisms is 0 or 1 (Bartlett, 1937). For a more detailed information on the arc sine transformation, see the Appendix.

12.5.5 The results of the analysis of variance of the data from Table 2 are shown in Table 3, and indicate a statistically significant difference in survival among NaPCP concentrations.

12.5.6 The results of the comparison of the control with the treatment effects, using 18 degrees of freedom and with a Dunnett's "t" value of 2.41 ($P = 0.05$), indicate that the NOEC is Concentration 5 (256 ug/L) and the LOEC is Concentration 6 (512 ug/L). The computer printout of these results is as follows:

```
THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 2
(32 UG NAPCP/L) AND CONTROL.

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 3
(64 UG NAPCP/L) AND CONTROL.

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 4
(128 UG NAPCP/L) AND CONTROL.

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 5
(256 UG NAPCP/L) AND CONTROL.

THERE IS A SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 6
(512 UG NAPCP/L) AND CONTROL.
```

12.5.7 For this set of data, the minimum difference that can be detected as statistically significant is 0.177. This represents a 19% reduction in the mean response (survival) from the control.

12.6 The chronic value (ChV) is the geometric mean of the NOEC and LOEC and is calculated as follows:

Log_{10} NOEC = Log_{10} 256 = 2.4082
Log_{10} LOEC = Log_{10} 512 = 2.7093
ChV = Antilog $(2.4082 + 2.7093)/2$ = Antilog 2.5588
ChV = 362 ug/L NaPCP

12.7 Analysis of Growth Data - Dunnett's Procedure

12.7.1 The use of Dunnett's Procedure to analyze the growth data, expressed in terms of average dry weight (Table 2), is similar to that for the survival data, except that the weight data are not transformed.

12.7.2. The average dry weight of the larvae from each replicate test chamber (Table 2) is entered into the program.

12.7.3. The results from the analysis of variance of the data in Table 2 are found in Table 4. The analysis indicates a statistically significant difference in the effects on larval growth at the various concentrations of NaPCP.

12.7.4. The results of the comparison of the average weights for each treatment with the average weights for the control, using 18 degrees of freedom and with a Dunnett's "t" value of 2.41 ($P = 0.05$), indicate that the NOEC is Concentration 4 (128 ug NaPCP/L) and the LOEC is Concentration 5 (256 ug NaPCP/L). The computer output from the Dunnett program is as follows (the control is Concentration 1):

```
THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 2
(32 UG NAPCP/L) AND CONTROL.

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 3
(64 UG NAPCP/L) AND CONTROL.

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 4
(128 UG NAPCP/L) AND CONTROL.

THERE IS A SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 5
(256 UG NAPCP/L) AND CONTROL.

THERE IS A SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 6
(512 UG NAPCP/L) AND CONTROL.
```

For this set of data, the minimum difference that can be detected as statistically significant is 0.095. This represents a 13.3% reduction in the mean response from the control.

12.6.5 The chronic value (ChV) is the geometric mean of the NOEC and LOEC and is calculated as follows:

$\mathrm{Log}_{10}\ \mathrm{NOEC} = \mathrm{Log}_{10}\ 128 = 2.1072$

$\mathrm{Log}_{10}\ \mathrm{LOEC} = \mathrm{Log}_{10}\ 256 = 2.4082$

$\mathrm{ChV} = \mathrm{Antilog}\ (2.1072 + 2.4082)/2 = \mathrm{Antilog}\ 2.2577$

ChV = 181 ug/L NaPCP

12.6.6 The results of the test indicate that growth was a more sensitive index of the effects of NaPCP than was survival.

13. PRECISION AND ACCURACY

13.1 PRECISION

13.1.1 Information on the single laboratory precision of the fathead minnow larval survival and growth test is presented in Table 5.

13.2 ACCURACY

13.2.1 The accuracy of toxicity tests can not be determined.

TABLE 1. SUMMARY OF RECOMMENDED TEST CONDITIONS FOR FATHEAD MINNOW (PIMEPHALES PROMELAS) LARVAL SURVIVAL AND GROWTH TEST

1. Test type:	Static renewal
2. Temperature (°C):	25 ± 2°C
3. Light quality:	Ambient laboratory illumination
4. Light intensity:	10-20 uE/m^2/s (50-100 ft-c)(ambient lab levels)
5. Photoperiod:	16 h light, 8 h darkness
6. Test chamber size:	1-L containers
7. Test solution volume:	500 mL/replicate
8. Renewal of test concentrations:	Daily
9. Age of test organisms:	Newly hatched larvaed
10. Larvae/test chamber and control:	10 larvae/chamber; Minimum of 20 larvae/test concentration
11. Replicate chambers/concentration:	Minimum of 2
12. Feeding regime:	Feed 0.1 mL newly hatched brine shrimp nauplii three times daily, 4 h between feedings (at the begining, midway, and the end of the work day)
13. Cleaning:	Siphon daily, immediately before test solution renewal
14. Aeration:	None, unless DO concentration falls below 40% saturation. Rate should be less than 100 bubbles/min.
15. Dilution water:	Moderately hard standard water, receiving water, other surface water, ground water, or synthetic water similar to receiving water
16. Effluent concentrations:	At least 5 and a control
17. Dilution factor:	Approximately 0.3 or 0.5
18. Test duration:	7 days
19. Effects measured:	Survival and growth (increase in weight)

TABLE 2. SUMMARY OF SURVIVAL AND GROWTH DATA OF FATHEAD MINNOW LARVAE EXPOSED FOR SEVEN DAYS TO SODIUM PENTACHLOROPHENATE

Test Conc. No.	NaPCP Conc. (ug/L)	Proportion of Survival in Replicate Chambers				Mean Prop. Surv.	CV[a] (%)	Ave Dry Wgt (mg) In Replicate Chambers				Mean Dry Wgt (mg)	CV (%)
		A	B	C	D			A	B	C	D		
1	Control	1.0	1.0	0.9	0.9	0.95	6	0.711	0.662	0.718	0.767	0.714	6
2	32	0.8	0.8	1.0	0.8	0.85	12	0.646	0.626	0.723	0.700	0.674	7
3	64	0.9	1.0	1.0	1.0	0.975	5	0.669	0.669	0.694	0.676	0.677	2
4	128	0.9	0.9	0.8	1.0	0.90	9	0.629	0.680	0.513	0.672	0.624	12
5	256	0.7	0.9	1.0	0.5	0.775	29	0.650	0.558	0.606	0.508	0.580[b]	11
6	512	0.4	0.3	0.4	0.2	0.325[b]	29	0.358	0.543	0.488	0.495	0.471[b]	17

[a] Coefficient of variation (standard deviation X 100/mean).

[b] Significantly different from control (P = 0.05).

TABLE 3. ANALYSIS OF VARIANCE OF SURVIVAL DATA IN TABLE 2.

Source	DF	Sum of Squares	Mean Square	Calculated F	Tabular $F_{0.05}$
Among	5	1.575	0.315	13.3[a]	2.77
Within	18	0.426	0.024		
Total	23	2.001			

[a] Significant at P = 0.01.

TABLE 4. RESULTS FROM ANALYSIS OF VARIANCE OF DRY WEIGHT DATA IN TABLE 2.

Source	DF	Sum of Squares	Mean Square	Calculated F	Tabular $F_{0.05}$
Among	5	0.155	0.031	9.31[a]	2.77
Within	18	0.060	0.003		
Total	23	0.215			

[a] Significant at P = 0.05

TABLE 5. PRECISION OF THE FATHEAD MINNOW LARVAL SURVIVAL AND GROWTH TEST, USING NAPCP AS A REFERENCE TOXICANT[a]

Test	NOEC (ug/L)	LOEC (ug/L)	Chronic Value (ug/L)
1	256	512	362
2	128	256	181
3	256	512	362
4	128	256	181
5	128	256	181
6	256	512	362

[a] For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

SECTION 12

TEST METHOD[1],[2]

FATHEAD MINNOW (PIMEPHALES PROMELAS) EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST

METHOD 1001.0

1. SCOPE AND APPLICATION

1.1 This method estimates the chronic toxicity of whole effluents and receiving water to the fathead minnow (*Pimephales promelas*), using embryos and larvae in an eight-day, static renewal test. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms. The test is useful in screening for teratogens because organisms are exposed during embryonic development.

1.2 Detection limits of the toxicity of an effluent or pure substance are organism dependent.

1.3 Single or multiple excursions in acute toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly volatile and highly degradeable toxicants in the source may not be detected in the test.

1.4 This method should be restricted to use by or under the supervision of professionals experienced in aquatic toxicity testing.

2. SUMMARY OF METHOD

2.1 Fathead minnow embryos and larvae are exposed in a static renewal system, from shortly after fertilization of the eggs through four days posthatching (total of eight days), to different concentrations of effluent or to receiving water. Test results are based on the total frequency of both mortality and gross morphological deformities (terata).

3. DEFINITIONS

(Reserved for addition of terms at a later date.)

4. INTERFERENCES

4.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities and Equipment).

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[1] The format used for this method was taken from Kopp, 1983.

[2] This method was adapted from Birge and Black, 1981.

4.2 Adverse effects of low dissolved oxygen (DO), high concentrations of suspended and/or dissolved solids, and extremes of pH may mask the presence of toxic substances.

4.3 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling and Sample Handling).

4.4 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival, and confound test results.

5. SAFETY

5.1 See Section 3, Health and Safety.

6. APPARATUS AND EQUIPMENT

6.1 Laboratory fathead minnow and brine shrimp culture units -- See Peltier and Weber, 1985. To test effluent toxicity on-site or in the laboratory, sufficient numbers of newly fertilized eggs must be available, preferably from a laboratory fathead minnow culture unit. If necessary, embryos can be shipped in well oxygenated water in insulated containers.

6.2 Samplers -- automatic sampler, preferrably with sample cooling capability, that can collect a 24-h composite sample of 2 L.

6.3 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling and Sample Handling).

6.4 Environmental chamber or equivalent facility with temperature control (25± 2°C).

6.5 Water purification system -- Millipore Super-Q or equivalent.

6.6 Balance -- analytical, capable of accurately weighing 0.0001 g.

6.7 Reference weights, Class S -- for checking performance of balance.

6.8 Test chambers -- borosilicate glass or non-toxic plastic labware; a minimum of two 500 mL beakers or deep petri dishes with covers per test concentration. The chambers should be covered during the test to avoid potential contamination from the air. Care must be taken to avoid inadvertently removing embryos or larvae when test solutions are decanted from the chambers. The covers are removed only for observation and removal of dead organisms.

6.9 Dissecting microscope, or long focal length magnifying lens, hand or stand supported -- for examining embryos and larvae in the test chambers.

6.10 Light box, microscope lamp, or flashlight -- for illuminating embryos and chambers during examination and observation of embryos and larvae.

6.11 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL, for making test solutions.

6.12 Volumetric pipets-- Class A, 1-100 mL.

6.13 Serological pipets-- 1-10 mL, graduated.

6.14 Pipet bulbs and fillers -- Propipet^R, or equivalent.

6.15 Droppers, and glass tubing with fire polished edges, 2-mm ID -- for transferring embryos, and 4-mm ID -- for transferring larvae.

6.16 Wash bottles -- for washing embryos from substrates and containers and for rinsing small glassware and instrument electrodes and probes.

6.17 Glass or electronic thermometers -- for measuring water temperatures.

6.18 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

6.19 National Bureau of Standards Certified thermometer (see EPA Method 170.1, USEPA 1979b).

6.20 pH, DO, and specific conductivity meters -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of the above parameters, a portable, field-grade instrument is acceptable.

6.21 Miscellaneous apparatus and equipment -- transfer containers, pumps, and automatic dilution devices should be constructed of materials as indicated in Section 5, Facilities and Equipment.

7. REAGENTS AND CONSUMABLE MATERIALS

7.1 Reagent water -- defined as carbon-filtered distilled or deionized water which does not contain substances which are toxic to the test organisms. A water purification system may be used to generate reagent water (see paragraph 6.5 above).

7.2 Effluent, surface water, and dilution water -- see Section 7, Dilution Water, and Section 8, Effluent and Surface Water Sampling and Sample Handling.

7.3 Reagents for hardness and alkalinity tests (see EPA Methods 130.2 and 310.1, USEPA 1979b).

7.4 pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) for standards and calibration check (see USEPA Method 150.1, USEPA 1979b).

7.5 Membranes and filling solutions for dissolved oxygen probe (see USEPA Method 360.1, USEPA 1979b), or reagents for modified Winkler analysis.

7.6 Laboratory quality assurance samples and standards for the above methods.

7.7 Specific conductivity standards (see EPA Method 120.1, USEPA 1979b).

7.8 Reference toxicant solutions (see Section 4, Quality Assurance).

7.9 Test Organisms -- fathead minnow embryos, 2- to 24-h old (preferably less than 12-h old), and spawned over less than an 8-h period, are used for the test (for fathead minnow culturing methods (Peltier and Weber, 1985). Spawning substrates with the newly-spawned, fertilized embryos are removed from the spawning tanks or ponds, and the embryos are separated from the spawning substrate by using the index finger and rolling the embryos gently with a circular movement of the finger (See Gast and Brungs, 1973). The embryos are then washed from the spawning substrate into a petri dish, using the spray from a wash bottle filled with fresh culture water, and are examined using a dissecting microscope or other suitable magnifying device. Damaged and infertile eggs are discarded. It is strongly recommended that the embryos be obtained from fish cultured inhouse, rather than from outside sources, to eliminate the uncertainty of damage caused by shipping and handling that may not be observable, but which might affect the results of the test.

7.9.1 The embryos from several substrates are then pooled in a single container to provide a sufficient number to conduct the required number of tests. These embryos may be used immediately to start a test or may be placed in a suitable container and transported for use at a remote location. When overnight transportation is required, embryos should be obtained when they are 6- to 8-h old. This permits the tests at the remote site to be started with less than 24-h old embryos. Embryos should be transported or shipped in clean, opaque, insulated containers, in well aerated or oxygenated fresh culture or dilution water, and should be protected from extremes of temperature and any other stressful conditions during transport. Instantaneous changes of water temperature when embryos are transferred from culture unit water to test dilution water, or from transport container water to on-site test dilution water, should be less than 2^{o}C. Instantaneous changes of pH, dissolved ions, osmotic strength, and DO should also be kept to a minimum.

7.9.2 The number of embryos needed to start the test will depend on the number of tests to be conducted and the objectives. The test is usually conducted with at least duplicate test chambers at each toxicant concentration and in the control. At least 40 embryos are exposed per test concentration (20 embryos in each of duplicate test chambers), although a larger number, such as 100 (50 embryos in each of duplicate test chambers), will provide better precision. If fewer than 40 embryos are used per concentration, test precision will be significantly reduced. Using more than 100 embryos per test concentration will not provide sufficient advantage to offset the increased time and effort required for observation and counting and removal of dead embryos during the test. The use of 50 embryos per replicate and 100 embryos per test concentration (a total of 600 embryos for five test concentrations and a control) is considered optimal.

8. SAMPLE COLLECTION, PRESERVATION AND HANDLING

8.1 See Section 8, Effluent and Receiving Water Sampling and Sample Handling.

9. CALIBRATION AND STANDARDIZATION

9.1 See Section 4, Quality Assurance.

10. QUALITY CONTROL

10.1 See Section 4, Quality Assurance.

11. PROCEDURES

11.1 TEST SOLUTIONS

11.1.1 Surface Waters

11.1.1.1 Surface water toxicity is determined with samples used directly as collected.

11.1.2 Effluents

11.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. One of two dilution factors, approximately 0.3 or 0.5, is commonly used. A dilution factor of approximately 0.3 allows testing between 100% and 1% effluent using only five effluent concentrations (100%, 30%, 10%, 3%, and 1%). This series of dilutions minimizes the level of effort, but because of the wide interval between test concentrations provides poor test precision (± 300%). A dilution factor of 0.5 provides greater precision (± 100%), but requires several additional dilutions to span the same range of effluent concentrations. Improvements in precision decline rapidly as the dilution factor is increased beyond 0.5

11.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 10%, 3%, 1%, 0.3%, and 0.1%). If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions at the lower range of effluent concentrations can be added.

11.1.2.3 The volume of effluent required for daily renewal of two replicates per concentration, each containing 400 mL of test solution, is approximately 2 L. Prepare enough test solution (approximately 1200 mL) at each effluent concentration to provide 400 mL additional volume for chemical analyses.

11.1.2.4 The hardness of the test solutions must exceed 25 mg/L ($CaCO_3$) to insure hatching success.

11.2 START OF THE TEST

11.2.1 Tests should begin as soon as possible, preferably within 24 h of sample collection. If the persistence of the sample toxicity is not known, the maximum holding time should not exceed 36 h. In no case should the test be started more than 72 h after sample collection. Just prior to testing, the temperature of the sample should be adjusted to 25 + 2°C and maintained at that temperature until portions are added to the dilution water.

11.2.2 Gently agitate and mix the embryos to be used in the test in a large container so that eggs from different spawns are evenly dispersed.

11.2.3 Randomly add the embryos to each test chamber. Accomplish this by adding a small number of embryos (5 to 15) to test chambers selected in a random fashion using a small bore (2mm) glass tube calibrated to contain approximately the desired number of embryos. Repeat the process until the appropriate number of embryos have been added to each chamber. Alternately, the total number of embryos are added at the same time to randomly selected test chambers.

11.2.4 After the embryos have been distributed to each test chamber, examine and count them. Remove and discard damaged or infertile eggs and replace with new undamaged embryos. It may be more convenient and efficient to transfer embryos to intermediate containers of dilution water for examination and counting. After the embryos have been examined and counted in the intermediate container, assign them to the appropriate test chamber and transfer them with a minimum of dilution water. If more than one test is being performed, the exposure is started for all of the tests at approximately the same time.

11.2.5 Randomize the position of the test chambers at the beginning of the test.

11.3 LIGHT, PHOTOPERIOD AND TEMPERATURE

11.3.1 The light quality and intensity should be at ambient laboratory levels, approximately 10-20 $uE/m^2/s$, or 50 to 100 foot candles (ft-c), with a photoperiod of 16 h of light and 8 h of darkness. The test water temperature should be maintained at 25 ± 2°C.

11.4 DISSOLVED OXYGEN (DO)

11.4.1 Aeration may affect the toxicity of effluents and should be used only as a last resort to maintain satisfactory DO concentrations. The DO concentrations should not fall below 40% saturation. If it is necessary to aerate, all concentrations and the control should be aerated. The rate should not exceed 100 bubbles/min, using a pipet with a 1-2 mm orifice, such as a 1-mL Kimax Serological Pipet No. 37033, or equivalent. Care should be taken to ensure that turbulence resulting from the aeration does not cause undue physical stress to the fish.

11.5 FEEDING

11.5.1 Since feeding is not required, it is not necessary to clean the test chambers daily.

11.6 TEST SOLUTION RENEWAL

11.6.1 The test solutions are renewed daily using freshly collected samples, immediately after cleaning the test chambers. During the daily renewal process 7-10 mm of water is left in the chamber to ensure that the embryos and larvae remain submerged during the renewal process. New test solution (400 mL) should be added slowly by pouring down the side of the test chamber to avoid excessive turbulence for the larvae.

11.7 ROUTINE CHEMICAL AND PHYSICAL DETERMINATIONS

11.8.1 At a minimum, the following measurements are made:

11.8.1.1 DO is measured at the beginning and end of each 24-h exposure period at all test concentrations and in the control.

11.8.1.2 Temperature, pH, and conductivity are measured at the beginning of each 24-h exposure period at all test concentrations and in the control.

11.8.1.3 Alkalinity and hardness are measured at the beginning of each 24-h exposure period in 100% effluent and in the control.

11.8 OBSERVATIONS DURING THE TEST

11.8.1 At the end of the first 24 h of exposure, before renewing the test solutions, examine and count the embryos. Remove the dead embryos (milky colored and opaque) and record the number. If the rate of mortality or fungal infection exceeds 20% in the control chambers, or if excessive non-concentration-related-mortality occurs, terminate the test and start a new test with new embryos. If the above mortality conditions do not occur, continue the test for the full eight days.

11.8.2 At 25°C, hatching begins on about the fourth day. After hatching begins, count the number of dead and live embryos and the number of hatched, dead, live, and deformed larvae, daily. Deformed larvae are those with gross morphological abnormalities such as lack of appendages, lack of fusiform shape (non-distinct mass), lack of mobility, a colored, beating heart in an opaque mass, or other characteristics that preclude survival. Remove dead embryos and larvae as previously discussed and record the numbers for all of the test observations (see sample data record in Table 1 below).

11.8.3 Protect the embryos and larvae from unnecessary disturbance during the test by carrying out the daily test observations, solution renewals, and removal of dead organisms, carefully. Make sure the test organisms remain immersed during the performance of the above operations.

11.9 TERMINATION OF THE TEST

11.9.1 The test is terminated after eignt days of exposure. Count the number of surviving, dead, and deformed larvae, and record the numbers of each. The deformed larvae are treated as dead. Keep a separate record of the total number of deformed larvae for use in reporting the teratogenicity of the test solution.

11.10 ACCEPTABILITY OF TEST RESULTS

11.10.1 For the test results to be acceptable, survival in the controls must be at least 80%, except where survival in any test concentration is 80% or better.

11.11 SUMMARY OF TEST CONDITIONS

11.11.1 A summary of test conditions is listed in Table 1.

12. CALCULATIONS

12.1 The endpoints of this toxicity test are based on the effects on survival and occurrence of terta. Table 2 shows the data on survival and deformaties after eight days of exposure to trickling filter waste.

12.2 The mortality data are analyzed using Probit Analysis (Finney, 1971), Dunnett's Procedure (Dunnett, 1955), or Steel's Many-One Rank Test (Steel, 1959; Miller, 1981).

12.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. Dunnett's Procedure is used when the tests for the assumptions described in the Appendix are satisfied. If the assumptions are not met, Steel's Many-One Rank Test may be more appropriate, but the advice of a statistician should be sought.

12.4 Data Preparation

12.4.1 Total mortality (combined total number of dead embryos, dead larvae, and deformed larvae) has been found to be the most sensitive endpoint. Tabulate and summarize the data and combine the number of dead embryos, dead larvae, and deformed larvae at each concentration. Total mortality is expressed as the mortality proportion, and can be determined by dividing the total mortality by the number of live embryos at the beginning of the test.

12.5 DATA ANALYSIS

12.5.1 Probit Analysis

12.5.1.1 Probit Analysis is used to determine the concentration causing 1% mortality. In this analysis, the mortality data from the test replicates at a given concentration are combined (see Table 2B).

The program transforms the concentration values to $\log_{10}$ and the percent mortality to probits, and then performs a regression analysis. A listing of the computer program and an example of data input and program output for the Probit Analysis are provided in the Appendix.

12.5.1.2 Report the LC1 and its 95% confidence limits. The LC1 is an estimate of the threshold (chronically toxic) concentration. For the sample data in Table 2, the LC1 is 4.7% effluent, with upper and lower 95% confidence intervals of 5.6% and 3.6% effluent, respectively.

12.5.1.3 If an LC1 can not be calculated using Probit analysis, analyze the data using Dunnett's Procedure, as described below.

12.5.2 Dunnett's Procedure

12.5.2.1 Dunnett's Procedure includes an analysis of variance (ANOVA) followed by a comparison of each toxicant concentration mean with the control mean. The error value calculated in the ANOVA is used in the comparison of the control and treatment means. An example of Dunnett's Procedure is included in the Appendix. The computer program listed in the Appendix generates output that includes an ANOVA table, a statement about each treatment mean that can be used to identify the NOEC, and the minimum difference between treatment and control means that can be detected as statistically significant.

12.5.2.2 It is necessary to have at least duplicate test chambers at each treatment concentration to carry out Dunnett's Procedure. With Steel's Many-One Rank Test, it is necessary to have at least four replicates per treatment.

12.5.2.3 The computer program makes the necessary transformation of the survival data by converting the square root of the proportion of surviving organisms to arc sine during the analysis, and includes a special modification of the arc sine transformation which is required where the proportion of surviving organisms is 0 or 1 (Bartlett, 1937). For a more detailed information on the arc sine transformation see the Appendix.

12.5.2.4 The results of the computer analysis of the data in Table 2A using by Dunnett's Procedure is provided below. The results of the analysis of variance are shown in Table 3, and indicate a statistically significant difference in survival among effluent concentrations. The results of the comparisons of the control mean with the treatment means is as follows:

```
THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 2
(3% EFFLUENT) AND CONTROL.

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 3
(5% EFFLUENT) AND CONTROL.

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 4
(7% EFFLUENT) AND CONTROL.
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THERE IS A SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 5
(11% EFFLUENT) AND CONTROL.

THERE IS A SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 6
(16% EFFLUENT) AND CONTROL.

12.5.2.5 Report the NOEC and LOEC. For the set of data analyzed, the NOEC is 7% effluent and the LOEC is 11% effluent.

12.5.2.6 For this set of data, the minimum difference that can be detected as statistically significant is 0.2385. This represents a 28.7% change in the response from the control.

12.5.3 The chronic value (ChV) is the geometric mean of the NOEC and LOEC and is calculated as follows:

Log_{10} NOEC = Log_{10} 7.0 = 0.8451
Log_{10} LOEC = Log_{10} 11.0 = 1.0414
ChV = Antilog (0.8451 + 1.0414)/2 = Antilog 0.9432
ChV = 8.8% effluent

13. PRECISION AND ACCURACY

13.1 PRECISION

13.1.1 Data shown in Tables 4 and 5 indicate that the precision of the embryo-larval survival and teratogenicity test, expressed as the relative standard deviation (or coefficient of variation, CV) of the LC1 values, was 62% for cadmium (Table 4), and 41% for Diquat (Table 5).

13.1.2 Precision data are also available from four embryo-larval survival and teratogenicity tests on trickling filter pilot plant effluent (Table 6). Although the data could not be analyzed by Probit Analysis, the NOECs and LOECs obtained using Dunnett's Test were the same for all four tests, 7% and 11% effluent, respectively, indicating maximum precision in terms of the test design.

13.2 ACCURACY

13.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 1. SUMMARY OF RECOMMENDED TEST CONDITIONS FOR THE FATHEAD MINNOW (PIMEPHALES PROMELAS) EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST

1. Test type:	Static renewal
2. Temperature:	25 ± 2°C
3. Light quality:	Ambient laboratory light
4. Light intensity:	10-20 uE/m^2/s, or 50-100 ft-c (ambient laboratory levels)
5. Photoperiod:	16 h light, 8 h dark
6. Test chamber size:	500 mL
7. Test solution volume:	400 mL
8. Renewal of test concentration:	Daily
9. Age of test organisms:	2- to 24-h old embryos; preferably less than 12-h old
10. No. of embryos/chamber:	20 to 50
11. Replicate test chambers/concentration:	2
12. Embryos per concentration:	40 to 100
13. Feeding regime:	Feeding not required
14. Aeration:	None unless DO falls below 40% saturation
15. Dilution water:	Hardness greater than 25 mg/L ($CaCO_3$); receiving water or other surface water, ground water, or synthetic water if similar to receiving water
16. Effluent test concentrations:	5 and a control
17. Dilution factor:	Approximately 0.3 or 0.5
18. Test duration:	8 days
19. Effects measured:	Percent hatch, percent larvae with terata, percent of normal larvae surviving 4 days post-hatch; surviving normal larvae from original embryos

TABLE 2. DATA FROM FATHEAD MINNOW EMBRYO-LARVAL TOXICITY TEST WITH TRICKLING FILTER WASTE

A. REPLICATES A AND B (USED IN DUNNETT'S PROCEDURE)

Repl.	Effl. Conc. (%)	No. Eggs at Start	Dead at Hatching No.	(%)	Dead + Deform. at hatching No.	(%)	Dead at Test Termination No.	(%)	Dead + Deform. at termination No.	(%)
A	Cont.	51	5	10	6	12	6	12	7	14
	3	50	5	10	5	10	5	10	5	10
	5	52	5	10	6	12	5	10	6	12
	7	50	2	4	8	16	9	18	15	30
	11	50	10	20	25	50	17	34	32	64
	16	49	39	80	39	80	49	100	49	100
B	Cont.	49	9	18	9	18	10	20	10	20
	3	50	6	12	6	12	9	18	9	18
	5	50	10	20	10	20	10	20	10	20
	7	50	6	12	10	20	16	32	20	40
	11	49	30	61	37	76	33	66	40	82
	16	50	29	58	34	68	45	90	50	100

B. COMBINED DATA FROM REPLICATES A AND B (USED IN PROBIT ANALYSIS)

Repl.	Effl. Conc. (%)	No. Eggs at Start	Dead at Hatching No.	(%)	Dead + Deform. at hatching No.	(%)	Dead at Test Termination No.	(%)	Dead + Deform. at termination No.	(%)
A&B	Cont.	100	14	14	15	15	16	16	17	17
	3	100	11	11	11	11	14	14	14	14
	5	102	15	15	16	16	15	15	16	16
	7	100	8	8	18	18	25	25	35	35
	11	99	40	40	62	62	50	50	72	73
	16	99	68	69	73	74	94	95	99	100

TABLE 3. ANALYSIS OF VARIANCE TOTAL MORTALILTY DATA IN TABLE 2A, BASED ON THE PROPORTION OF DEAD AND DEFORMED ORGANISMS IN REPLICATES A AND B AT TEST TERMINATION

Source	DF	Sum of Squares	Mean Square	Calculated F	Tabular $F_{0.05}$
Among	5	2.089	0.418	58.80[a]	4.39
Within	6	0.043	0.007		
Total	11	2.132			

[a] Significant at P = 0.05.

TABLE 4. PRECISION OF THE FATHEAD MINNOW EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY TEST, USING CADMIUM AS A REFERENCE TOXICANT[a,b]

Test	LC1[c] (mg/L)	95% Confidence Limits	NOEC[d] (mg/L)
1	0.014	0.009 - 0.018	0.012
2	0.006	0.003 - 0.010	0.012
3	0.005	0.003 - 0.009	0.013
4	0.003	0.002 - 0.004	0.011
5	0.006	0.003 - 0.009	0.012
N	5		
Mean	0.0068		
SD	0.0042		
CV(%)	62		

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[a] Tests conducted by Drs. Wesley Birge and Jeffrey Black, University of Kentucky, Lexington, under a cooperative agreement with the Aquatic Biology Section, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio (Cornelius I. Weber, Project Officer).

[b] Cadmium chloride was used as the reference toxicant. The nominal concentrations, expressed as cadmium (mg/L), were: 0.01, 0.032, 0.100, 0.320, and 1.000. The water hardness was 100 mg/L as calcium carbonate.

[c] Determined by Probit Analysis.

[d] Highest no-observed-effect concentration determined by independent statistical analysis (2x2 Chi-square Fisher's Exact Test).

TABLE 5. PRECISION OF THE FATHEAD MINNOW, EMBRYO-LARVAL, SURVIVAL AND TERATOGENICITY TOXICITY TEST, USING DIQUAT AS A REFERENCE TOXICANT[a]

Test	LC1[b] (mg/L)	95% Confidence Limits
1	0.58	0.32 - 0.86
2	2.31	c
3	1.50	1.05 - 1.87
4	1.71	1.24 - 2.09
5	1.43	0.93 - 1.83
N	5	
Mean	1.51	
SD	0.62	
CV(%)	41.3	

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[a] Tests conducted by Drs. Wesley Birge and Jeffrey Black, University of Kentucky, Lexington, under a cooperative agreement with the Aquatic Biology Section, Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio (Cornelius I. Weber, Project Officer).

[b] Determined by Probit analysis.

[c] Not calculatable.

TABLE 6. PRECISION OF FATHEAD MINNOW EMBRYO-LARVAL SURVIVAL AND TERATOGENICITY STATIC-RENEWAL TEST CONDUCTED WITH TRICKLING FILTER EFFLUENT[a,b]

Test No.	NOEC (% Effl)	LOEC (% Effl)
1	7	11
2	7	11
3	7	11
4	7	11

[a] Effluent concentrations used: 3, 5, 7, 11 and 16%.

[b] Maximum precision achieved in terms of NOEC-LOEC interval. For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

SECTION 13

TEST METHOD[1,2]

CERIODAPHNIA SURVIVAL AND REPRODUCTION TEST
METHOD 1002.0

1. SCOPE AND APPLICATION

1.1 This method measures the chronic toxicity of whole effluents and receiving water to the cladoceran, *Ceriodaphnia dubia*, during a seven-day, static renewal exposure. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

1.2 Detection limits of the toxicity of an effluent or pure substance are organism dependent.

1.3 Single or multiple excursions in acute toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling and because the test chambers are not sealed, highly volatile and highly degradeable toxicants in the source may not be detected in the test.

1.4 This method should be restricted to use by or under the supervision of professionals experienced in aquatic toxicity testing.

2. SUMMARY OF METHOD

2.1 *Ceriodaphnia* are exposed in a static renewal system for seven days to different concentrations of effluent, or to receiving water. Test results are based on survival and reproduction. If the test is conducted as described, the control organisms should produce three broods of young during the seven-day period.

3. DEFINITIONS

(Reserved for addition of terms at a later date.)

4. INTERFERENCES

4.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities and Equipment).

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[1] The format used for this method was taken from Kopp, 1983.

[2] This method was adapted from Norberg and Mount, 1984.

4.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling and Sample Handling).

4.3 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival, and confound test results.

4.4 The amount and type of natural food in the effluent or dilution water may confound test results.

4.5 Food added during the test may sequester metals and other toxic substances and confound test results.

5. SAFETY

5.1 See Section 3, Health and Safety.

6. APPARATUS AND EQUIPMENT

6.1 Laboratory *Ceriodaphnia* culture unit -- See culturing methods below. To test effluent or receiving water toxicity, sufficient numbers of newborn (neonate) organisms must be available.

6.2 Samplers -- Automatic sampler, preferrably with sample cooling capability, capable of collecting a 24-h composite sample of 1 L.

6.3 Sample containers -- for sample shipment and storage (See Section 8, Effluent and Receiving Water Sampling and Sample Handling).

6.4 Environmental chamber, incubator, or equivalent facility with temperature control (25± 1°C).

6.5 Water purification system -- Millipore Super-Q or equivalent.

6.6 Balance -- Analytical, capable of accurately weighing 0.0001 g.

6.7 Reference weights, Class S -- for checking performance of balance.

6.8 Racks for test vessels -- Racks approximately 8 cm x 40 cm, drilled to hold 10 test vessels each.

6.9 Dissecting microscope -- for examining organisms in the test chambers.

6.10 Light box -- for illuminating organisms during examination.

6.11 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL, for culture work and preparation of test solutions.

6.12 Volumetric pipets-- Class A, 1-100 mL.

6.13 Serological pipets-- 1-10 mL, graduated.

6.14 Pipet bulbs and fillers -- Propipet^R, or equivalent.

6.15 Disposable polyethylene pipets, droppers, and glass tubing with fire-polished edges, 2-mm ID -- for transferring organisms.

6.16 Wash bottles -- for rinsing small glassware and instrument electrodes and probes.

6.17 Glass or electronic thermometers -- for measuring water temperatures.

6.18 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

6.19 National Bureau of Standards Certified thermometer -- see EPA Method 170.1, USEPA 1979b.

6.20 pH, DO, and specific conductivity meters -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of the above parameters, a portable, field-grade instrument is acceptable.

6.21 Miscellaneous apparatus and equipment -- transfer containers, pumps, and automatic dilution devices should be constructed of materials as indicated in Section 5, Facilities and Equipment.

7. REAGENTS AND CONSUMABLE MATERIALS

7.1 Reagent water -- defined as activated-carbon-filtered distilled or deionized water which does not contain substances which are toxic to the test organisms. A water purification system may be used to generate reagent water (see paragraph 6.5 above).

7.2 Effluent, surface water, and dilution water -- see Section 7, Dilution Water, and Section 8, Effluent and Surface Water Sampling and Sample Handling. Dilution water that contains undesirable organisms, that may attack the test organisms should be filtered through a fine mesh net (30-um or smaller openings).

7.3 Reagents for hardness and alkalinity tests (see EPA Methods 130.2 and 310.1, USEPA 1979b).

7.4 pH buffers 4, 7, and 10 (or as per instructions of instrument manufacturer) for standards and calibration check (see USEPA Method 150.1, USEPA 1979b).

7.5 Membranes and filling solutions for dissolved oxygen probe (see USEPA Method 360.1, USEPA 1979b), or reagents for modified Winkler analysis.

7.6 Laboratory quality assurance samples and standards for the above methods.

7.7 Specific conductivity standards (see EPA Method 120.1, USEPA 1979b).

7.8 Reference toxicant solutions (see Section 4, Quality Assurance).

7.9 Test Vessels -- 30-mL borosilicate glass beakers or disposable plastic salad dressing cups (manufactured by Anchor-Hocking Plastic Division, and supplied by Plastics Inc., 224 Ryan Avenue, St. Paul, Minnesota, 55164) are recommended because they will fit in the viewing field of most stereoscopic microscopes. Rinse thoroughly in distilled water and then in dilution water before use. A volume of 15 mL of test solution is adequate for the organisms, and will provide a depth in which it is possible to count the animals with a minimum of re-focusing. Ten test vessels are used for each effluent dilution and for the control.

7.10 Test Organisms -- Neonate Ceriodaphnia dubia 2- to 24-h old and released during the same 4-h period. See information on culturing methods below.

7.10.1 The test organism (species being used) cultures should be started at least two weeks before the brood animals are needed, to provide an adequate supply of neonates for the test. Only a few individuals are needed to start a culture because of their prolific reproduction.

7.10.2 Ceriodaphnia may be shipped or otherwise transported in polyethylene bottles. Several hundred animals will live as long as one week in a 1-L bottle filled three-fourths full with culture medium containing the trout chow diet (Paragraph 7.10.6.2.4 below). Animals received from an outside source should be transferred to new culture media gradually, over a period of 1-2 days, to avoid mass mortality.

7.10.3 It is best to start the culture from one animal, which is sacrificed after producing young, embedded, and retained as a permanent microscope slide mount to facilitate identification and permit future reference. The species identification of the stock culture should be verified by preparing slide mounts, regardless of the number of animals used to start the culture. The following procedure is recommended for making slide mounts of Ceriodaphnia (Beckett and Lewis, 1982):

1. Pipet the animal onto a watch glass.
2. Reduce the water volume by withdrawing excess water with the pipet.
3. Add a few drops of carbonated water (club soda or seltzer water) or 70% ethanol to relax the specimen so that the post-abdomen is extended. (Optional: with practice, extension of the postabdomen may be accomplished by putting pressure on the cover slip.)
4. Place a small amount (one to three drops) of mounting medium on a glass microscope slide. The recommended

mounting medium is CMCP-9/9AF Medium[1], prepared by mixing two parts of CMCP-9 with one part of CMCP-9AF. For more viscosity and faster drying, CMC-10 stained with acid fuchsin may be used.

5. Using a forceps or a pipet, transfer the animal to the drop of mounting medium on the microscope slide.
6. Cover with a cover slip and exert minimum pressure to remove any air bubbles trapped under the cover slip. Slightly more pressure will extend the postabdomen.
7. Allow mounting medium to dry.
8. Make slide permanent by placing CMC-10 around the edges of the coverslip.
9. Identify to species (see Pennak, 1978, and Berner, 1985).
10. Label with waterproof ink or diamond pencil.
11. Store for permanent record.

7.10.4 One-litre glass beakers are recommended for use as culture vessels. Use of aquaria (40- to 80-L, or 10- to 20-gal) and other types of culture vessels may also be convenient. Maintain cultures in several (four or more) separate vessels to provide back-up cultures in case one is lost due to accident or other problems, such as low DO concentrations or lack of food. Fill the 1-L culture vessels with 900 mL of medium.

7.10.5 A new culture is started each week, and the oldest culture is discarded. Using this schedule, 1-L cultures will provide 500 to 1000 neonate *Ceriodaphnia* per week for use in the tests.

7.10.6 Feeding

7.10.6.1 Feeding the proper amount of the right food is extremely important in *Ceriodaphnia* culturing. The key is to provide sufficient nutrition to support normal reproduction without adding excess food which may clog the animal's filtering apparatus or greatly decrease the DO concentration, and lead to the death of the animals. The suspension of trout chow, yeast, and CEROPHYL^R described below will provide adequate nutrition if fed daily at the rate of 3 mL/L of medium.

7.10.6.2 The combined food is prepared from three ingredients as follows:

7.10.6.2.1 Digested trout chow:

1. Add 5.0 g of No. 1 trout chow, U.S. Fish and Wildlife Service Specification Diet SD9-30, to 1 L of distilled or deionized water. This trout chow may be obtained from Ziegler Bros., Inc., P. O. Box 95, Gardners, PA, 17324. Mix well in a blender and aerate continuously (digest) for one week at ambient laboratory temperature prior to use.

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[1] CMCP-9 and 9AF are available from Polysciences, Inc., Paul Valley Industrial Park, Warrington, PA, 18976. (215-343-6484).

2. At the end of digestion period, place the mixture in a refrigerator and allow to settle overnight. Decant 300 mL of the supernatant and combine with equal volumes of supernatant from CEROPHYL^R and yeast preparations (below). Discard the remainder.

7.10.6.2.2 Yeast:

1. Add 5.0 g of dry yeast, such as FLEISCHMANN'S^R or ST. REGIS^R, to 1 L of distilled water.
2. Stir with a magnetic stirrer until well dispersed or use a blender at low speed for 5 min.
3. Place in a refrigerator overnite, mix well, and combine 300 mL with equal volumes of supernatant from the trout chow (above) and CEROPHYL^R preparations (below). Discard the remainder.

7.10.6.2.3 CEROPHYL^R (Powdered, Dried, Cereal Leaves)[1]:

1. Place 5.0 g of CEROPHYL^R powder in a blender.
2. Add 1 L of distilled water.
3. Mix at high speed for 5 min.
4. Place in a refrigerator overnite to settle, decant 300 mL of the supernatant and combine with equal volumes of supernatant from trout chow and yeast preparations (above). Discard the remainder.

7.10.6.2.4 Combined trout chow-yeast-CEROPHYL^R food:

1. Mix equal (300 mL) volumes of the three foods.
2. Place aliquots of the final mixture in small (50 mL to 100 mL) screw-cap plastic bottles and freeze until needed.
3. Fresh or thawed food is stored in the refrigerator between feedings, and is used for a maximum of one week.

7.10.6.2.5 The quality of each batch of food prepared with a new supply of components should be determined by using the food in a 7-day reproduction test with control water (use culture medium, see 7.10.7, below).

7.10.7 Synthetic, moderately hard water (hardness of 80 to 100 mg/L as $CaCO_3$) is recommended as a standard culture medium (see Table 1, Section 7, Dilution Water). Other culture water, such as well water, pond water, or dechlorinated tap water, also may be satisfactory.

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[1] Available from Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri, 63178. (800-325-3010).

7.10.8 *Ceriodaphnia* should be cultured at a temperature of 25 ± 2°C No water temperature control equipment is needed if the ambient laboratory temperature remains in the recommended range, and if there are no frequent, rapid, large, temperature excursions in the culture room.

7.10.9 Day/night cycles prevailing in most laboratories will provide adequate illumination for normal growth and reproduction. A 16-h/8-h day/night cycle is recommended.

7.10.10 *Ceriodaphnia* cannot survive DO concentrations below 5 mg/L for extended periods. However, aeration is generally not needed unless the cultures are overfed. DO should be measured when the cultures are first started and weekly thereafter. Aerate if the DO concentration drops below 5 mg/L.

7.10.11 Suspend a clear glass or plastic panel over the cultures to exclude dust and dirt. There should be space for circulation of air over the vessels to provide oxygen for the cultures.

7.10.12 *Ceriodaphnia* are eaten by many species of copepods and shrimp, and cultures must be maintained free of preditors. Natural waters used as culture media should be filtered through a plankton net with 30 um mesh openings.

7.10.13 *Ceriodaphnia* have been reported to be very sensitive to sudden pH and temperature changes, and care should be taken to limit rapid changes in pH to less than 0.5 units and rapid changes in water temperature to less than 5°C.

7.10.14 The test organisms should be handled carefully and as little as possible so that they are not unnecessarily stressed. They should be transferred from cultures to test vessels, and between test vessels, with a pipet of approximately 2-mm bore, taking care to release the animals under the surface of the water. Any organism that is injured during handling should be discarded.

7.10.15 Culture Maintenance

7.10.15.1 Cultures should be fed daily to maintain the organisms in optimum condition so as to provide maximum reproduction in the toxicity tests. Stock cultures which are stressed because they are not adequately fed may produce large number of males and ephippial females. Also, brood females and their offspring may produce few young when used in the test.

1. If food is frozen, remove a bottle of food from the freezer 1 h before feeding time, and allow to thaw.
2. Shake thoroughly.
3. Feed daily at the rate of 4 mL/L of medium.
4. Return unused food mixture to the refrigerator. Do not re-freeze. Discard unused portion after one week.

7.10.15.2 Careful culture maintenance is essential. The population should not be allowed to exceed 1000 animals/L of medium. If necessary, thin the cultures every four or five days after changing the medium to prevent crashes and discourage gametogenesis.

7.10.15.3 The culture medium in each culture vessel should be replaced with fresh medium weekly as follows:

1. Pour about one half (450 mL of the 900 mL) of the contents of a culture vessel into a shallow vessel. A large finger bowl works well.
2. Discard the remainder of the medium and animals unless needed for a test or to start a new culture.
3. Clean the culture vessel by brushing the sides and bottom, or wiping with a clean sponge or paper towel, and rinsing with distilled or deionized water. Each month, the culture vessels should be washed as described in Section 5, (Facilities and Equipment), and air dried.
4. Place about 100 mL of fresh medium in the clean culture vessel.
5. Remove about 100 *Ceriodaphnia* from the holding vessel (finger bowl) with a pipet, plastic tubing, or by dipping with a small beaker, and transfer them to the fresh medium, along with a small amount of the old medium to provide seed bacteria for the new culture.
6. Carefully add sufficient additional fresh medium to fill the culture vessel.

8. SAMPLE COLLECTION, PRESERVATION AND HANDLING

8.1 See Section 8, Effluent and Receiving Water Sampling and Sample Handling.

9. CALIBRATION AND STANDARDIZATION

9.1 See Section 4, Quality Assurance.

10. QUALITY CONTROL

10.1 See Section 4, Quality Assurance.

11. PROCEDURE

11.1 TEST SOLUTIONS

11.1.1 Surface Waters

11.1.1.1 Surface water toxicity is determined with samples used directly as collected.

11.1.2 Effluents

11.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. One of two dilution factors, approximately 0.3 or 0.5, is commonly used. A dilution factor of approximately 0.3 allows testing between 100% and 1% effluent using only five effluent concentrations (100%, 30%, 10%, 3%, and 1%). This series of dilutions minimizes the level of effort, but because of the wide interval between test concentrations provides poor test precision (± 300%). A dilution factor of 0.5 provides greater precision (± 100%), but requires several additional dilutions to span the same range of effluent concentrations. Improvements in precision decline rapidly as the dilution factor is increased beyond 0.5

11.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 10%, 3%, 1%, 0.3%, and 0.1%). If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions at the lower range of effluent concentrations can be added.

11.1.2.3 The volume of effluent required for daily renewal of 10 replicates per concentration, each containing 15 mL of test solution, is approximately 1 L. Prepare enough test solution (approximately 600 mL) at each effluent concentration to provide 400 mL additional volume for chemical analyses.

11.2 OBTAINING NEONATES FOR THE TEST

11.2.1 This test method requires neonates 2- to 24-h old (all within 4 h of the same age) to begin the test. To obtain a sufficient number of young which are all less than 4-h old, brood animals containing eggs are placed singly in 30-mL beakers containing 15 mL of media (using the same source of dilution water that will be used in the test), four or five days prior to the initiation of the test. One brood animal is needed for each test vessel that will be used in the test. For example, if five concentrations and a control will be used in a test to begin on a Friday, 60 brood animals are placed in individual beakers on Monday and fed daily, as in the test (see Paragraph 11.6). The brood stock are transferred to fresh media daily, and the young are discarded with the old media. Four hours before the test is to begin, the young are removed from the brood beakers and discarded. In this way, all the young in the brood beakers when the test is ready to start will be less than 4-h old, and are used in the test. Some workers prefer to begin removing and saving young from the brood chambers at 2- or 4-h intervals beginning 8 h before the test is scheduled to start. This makes it possible to use older, but similar aged, young for beginning the test, thus improving chances of good survival of test animals.

11.3 START OF THE TEST

11.3.1 The test should begin as soon as possible, preferably within 24 h of sample collection. In no case should the test be started more than 72 h after sample collection. Just prior to testing, the temperature of the sample should be adjusted to 25 ± 1°C and maintained at that temperature until portions are added to the dilution water.

11.3.2 Begin the test by randomly placing one neonate in each test beaker. Because of their small size and difficulty in handling, the test chambers are usually placed in racks, 10 to a rack. The position of the test chambers is randomized in the racks at the beginning of the test. On following days, the positions of the racks are randomized.

11.4 LIGHT, PHOTOPERIOD AND TEMPERATURE

11.4.1 The light quality and intensity should be at ambient laboratory levels, approximately 10-20 uE/m^2/s, or 50 to 100 foot candles (ft-c), with a photoperiod of 16 h of light and 8 h of darkness. It is critical that the test water temperature be maintained at 25 ± 1°C to obtain three broods in seven days.

11.5 DISSOLVED OXYGEN

11.5.1 Low DO concentrations may be important when running effluent toxicity tests. However, aeration is not practical for the *Ceriodaphnia* test. If the DO in the effluent and/or dilution water is low, aerate before preparing the test solutions.

11.6 FEEDING

11.6.1 During the test, the *Ceriodaphnia* are fed the same diet as used for the cultures. The organisms in the test vessels are fed digested trout chow-yeast-CEROPHYL^R diet daily at a rate of 0.1 mL food suspension/15 mL of test solution.

11.7 TEST SOLUTION RENEWAL

11.7.1 Using a glass or polyethylene dropper, or pipet, transfer each test organism daily to a new test vessel containing 15 mL of freshly-prepared test solution and 0.1 mL of the food suspension. The animals should be released under the surface of the water so that air is not trapped under the carapace.

11.8 ROUTINE CHEMICAL AND PHYSICAL DETERMINATIONS

11.8.1 At a minimum, the following measurements are made:

11.8.1.1 DO is measured in each test solution at the beginning of each 24-h exposure period, and at the end of the exposure period in one test vessel at each test concentrations and in the control, after the adult has been removed and the young have been counted. (Acid must not be added to count the young in that chamber.)

11.8.1.2 Temperature, pH, and conductivity are measured at the beginning of each 24-h exposure period at all test concentrations and in the control.

11.8.1.3 Alkalinity and hardness are measured at the beginning of each 24-h exposure period in 100% effluent and in the control.

11.9 OBSERVATIONS DURING THE TEST

11.9.1 Three broods are usually obtained in the controls in a seven-day test conducted at 25°C. The first brood of two to five young is usually released on the third day of the test, soon after the adults are transferred to fresh test solutions. Successive broods are released every 36 to 48 h thereafter, and may contain 10 to 15 young.

11.9.2 Each day, at the time the organisms are to be transferred to fresh test solutions, determine adult survival and count and record the number of young. First remove the adult to the new test solution. Count any dead young, and then add two drops of 1N hydrochloric acid to the vessel (except the vessel used for DO measurements). The living young die quickly and settle to the bottom of the test vessel where they may be counted with a minimum of effort and error. The young are discarded after counting.

11.9.3 The young are best counted with the aid of a stereomicroscope. The organisms are more easily seen if viewed against a black background. If counts are made without the aid of a stereomicroscope, place the test vessels on a black strip of tape on a light box.

11.9.4 In the absence of toxic substances, young production may exceed 30 per adult. If toxic substances are present, young may develop in the brood pouch of the adults, but may not be released during the exposure period.

11.10 TERMINATION OF THE TEST

11.10.1 Because of the rapid rate of development of *Ceriodaphnia*, the test must be terminated and all observations completed within ± 2 h of exactly seven days after the initiation of the test. The seven-day test period was selected because each control animal will normally produce three broods, totaling 10 to 30, at a test temperature of 25°C. An extension of more than a few hours in the test period would be a significant part of the brood production cycle of the animals, and could result in additional broods.

11.11 SUMMARY OF TEST CONDITONS

11.11.1 A summary of test conditions is listed in Table 1.

12. CALCULATIONS

12.1 DATA PREPARATION

12.1.1 The number of young produced per adult female is determined by taking the total number of young produced until either the time of death of the adult or the end of the experiment, whichever comes first. An animal that dies before producing young would be included in the analysis, with zero entered as the number of young produced. The subsequent calculation of the mean number of young produced per adult for each toxicant concentration provides a combined measure of the toxicant's effect on both mortality and reproduction.

12.1.2 Tabulate and summarize the data. A sample set of test data are listed in Table 2. Using these data, the total number of young produced per adult during the test is shown in Table 3.

12.2. DATA ANALYSIS

12.2.1 Fisher's Exact Test (Finney, 1948; Pearson and Hartley, 1962) and Dunnett's Procedure (Dunnett, 1955) or Steel's Many-One Rank Test (Steel, 1959; Miller, 1981) are used to analyze the data. The first step is to test for a significant difference in the survival of the original test organisms in the various effluent concentrations and control, using Fisher's Exact Test. Dunnett's Procedure is then used to analyze the reproduction data from only those effluent concentrations where mortality was not significantly different from the controls.

12.2.2 For the data in Table 3, reference to the tabulated values in Appendix E show that the survival in 25% effluent is significantly different from the control survival. Therefore, the 25% effluent concentration will not be included in the analysis of the reproduction data.

12.2.3 Since it is border-line as to whether the remaining data meet the assumptions for normality, both Dunnett's Procedure and Steel's Many-One Rank Test can be used to analyze the reproduction data.

12.2.4 The safe concentration derived from the test is reported as the NOEC.

12.2.5 Dunnett's Procedure

12.2.5.1 Dunnett's procedure includes an analysis of variance (ANOVA) followed by a comparison of each toxicant concentration mean with the control mean. The error value calculated in the ANOVA is used in the comparison of the control and treatment means. An example of a Dunnett's calculation is included in the Appendix. The computer program listed in the Appendix generates output which includes an ANOVA table, a statement about each treatment mean that can be used to identify the NOEC, and the minimum difference between treatment and control means that can be detected as statistically significant.

12.2.5.2 The analysis is carried out using the total number of live young produced by each adult female during the the test (Table 3).

12.2.5.3 The output from the analysis of variance is shown in Table 4, and indicates that the effects of the various treatments on reproduction were significant at $P = 0.05$.

12.2.5.4 The computer output for the comparison of the control mean with the treatment means is shown below:

```
THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 2
(1% EFFLUENT) AND CONTROL.

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 3
(3% EFFLUENT) AND CONTROL.

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 4
(6% EFFLUENT) AND CONTROL.

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 5
(12% EFFLUENT) AND CONTROL.
```

12.2.5.5 The NOEC and LOEC, as determined from the analysis, were 12% and 25% effluent, respectively. Note that the Dunnett program considers the control as Concentration 1. Thus, Concentration 5 in the output from Dunnett's test is effluent Concentration 4 (12% effluent) in the test.

12.2.5.6 For this set of data, the minimum difference that can be detected as statistically significant is 6.28 young per adult. This represents a 34.5% reduction in the response from the control.

12.2.6 Steel's Many-One Rank Test

12.2.6.1 Steel's Many-One Rank Test compares several treatments with a control by analyzing the ranks of the data, thereby eliminating the necessity that the data meet the normality assumptions. The calculations for Steel's Many-One Rank Test of the data in Table 3 are detailed in the Appendix. The NOEC determined from Steel's Test was 12%, which agrees with the NOEC from Dunnett's Procedure above.

12.2.7 The chronic value (ChV) is the geometric mean of the NOEC and LOEC and is calculated as follows:

$$\log_{10} \text{NOEC} = \log_{10} 12 = 1.0792$$
$$\log_{10} \text{LOEC} = \log_{10} 25 = 1.3979$$
$$\text{ChV} = \text{Antilog } (1.0792 + 1.3979)/2 = \text{Antilog } 1.2386$$
$$\text{ChV} = 17.3\% \text{ effluent}$$

13. PRECISION AND ACCURACY

13.1 PRECISION

13.1.1 Information on the single laboratory precision of the *Ceriodaphnia* reproduction test based on the NOEC and LOEC values from nine tests with the reference toxicant NaPCP is provided in Table 5.

13.2 ACCURACY

13.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 1. SUMMARY OF RECOMMENDED TEST CONDITIONS FOR *CERIODAPHNIA* SURVIVAL AND REPRODUCTION TEST

1. Test type:	Static renewal
2. Temperature (^oC):	25 ± 1°C
3. Light quality:	Ambient laboratory light
4. Light intensity:	10-20 $uE/m^2/s$, or 50-100 ft-c (ambient laboratory levels)
5. Photoperiod:	16 h light, 8 h dark
6. Test vessel size:	30 mL
7. Test solution volume:	15 mL
8. Renewal of test concentrations:	Daily
9. Age of test organisms:	Less than 24 h; and all released within a 4-h period
10. Number of test organisms per chamber:	1
11. Number of replicate chambers per treatment:	10
12. Feeding regime:	Feed 0.1 mL food suspension/15 mL, daily
13. Aeration:	None
14. Dilution water:	Moderately hard standard water, receiving water, other surface water, or ground water with hardness similar to receiving water
15. Dilution factor:	Approximately 0.3 or 0.5
16. Test duration:	7 days
17. Effects measured:	Survival and reproduction

TABLE 2. DATA FROM *CERIODAPHNIA* EFFLUENT TOXICITY TEST

Effl Conc	Day No.	Replicate										Total Live Young	No. Live Adults	Most Young By Any One Adult
		A	B	C	D	E	F	G	H	I	J			
Cont	3	0	0	0	0	0	x	0	0	0	0	0	9	0
	4	2	2	4	0	6	-	2	6	1	4	27	9	6
	5	9	2	9	0	9	-	0	6	2	9	46	9	9
	6	5	6	9	6	0	-	3	5	11	10	55	9	11
	7	6	8	5	10	1	-	3	8	10	3	54	9	10
		22	18	27	16	16	0	8	25	24	26	182		
1.0%	3	0	0	0	0	0	0	0	0	0	0	0	10	0
	4	2	4	1	0	4	2	2	3	2	2	22	10	4
	5	9	5	2	0	8	13	2	8	2	2	51	10	13
	6	6	3	6	2	8	8	10	0	6	6	55	10	10
	7	5	3	12	4	3	8	9	0	13	12	69	10	13
		22	15	21	6	23	31	23	11	23	22	197		
3.0%	3	0	0	0	0	0	0	0	0	0	0	0	10	0
	4	2	1	2	4	4	8	2	0	4	3	30	10	8
	5	3	4	2	9	11	6	2	4	8	6	55	10	11
	6	10	8	6	9	0	3	6	10	8	3	63	10	10
	7	10	12	10	2	0	6	11	8	0	6	65	10	12
		25	25	20	24	15	23	21	22	20	18	213		
6.0%	3	0	0	0	0	0	0	0	0	0	3	3	10	3
	4	2	4	2	2	0	1	2	0	4	2	19	10	4
	5	2	8	0	2	4	2	2	4	6	8	38	10	8
	6	9	2	2	3	10	6	8	6	2	4	52	10	10
	7	10	2	3	6	12	13	9	11	9	4	79	10	13
		23	16	7	13	26	22	21	21	21	21	191		
12.0%	3	0	0	0	0	0	0	0	0	0	0	0	10	0
	4	0	4	2	2	2	0	1	2	1	4	18	10	4
	5	1	8	0	2	3	3	0	2	3	0	22	10	8
	6	8	4	8	3	10	10	5	10	6	7	71	10	10
	7	11	5	7	6	10	12	10	11	8	7	87	10	12
		20	21	17	13	25	25	16	25	18	18	198		
25.0%	3	0	0	0	0	0	0	0	0	0	0	0	10	0
	4	x	0	0	0	x	x	0	x	0	0	0	6	0
	5	-	x	1^x	x	-	-	x	-	x	x	1	1	1
	6	-	-	-	-	-	-	-	-	-	-	0	0	0
	7	-	-	-	-	-	-	-	-	-	-	0	0	0
		0	0	1	0	0	0	0	0	0	0	1		

x = Dead adult, no young produced before death.

1^x = Dead adult; one young produced before death.

Note: Days 1 and 2 are not included because young were not produced until the third day. Adult mortality was not recorded for days 1 and 2.

TABLE 3. NUMBER OF YOUNG PRODUCED PER CERIODAPHNIA

Replicate (Organism)	Control	Effluent Concentration (%) 1	3	6	12	25
A	22	22	25	23	20	0
B	18	15	25	16	21	0
C	27	21	20	7	17	1
D	16	6	24	13	13	0
E	16	23	15	26	25	0
F	0	31	23	22	25	0
G	8	23	21	21	16	0
H	25	11	22	21	25	0
I	24	23	20	21	18	0
J	26	22	18	21	18	0

TABLE 4. ANALYSIS OF VARIANCE OF CERIODAPHNIA REPRODUCTION DATA FROM CONTROL AND FIRST FOUR EFFLUENT CONCENTRATIONS (1-12%)

Source	DF	Sum of Squares	Mean Square	Calculated F	Tabular $F_{0.05}$
Among	4	51.480	12.870	0.35	2.58
Within	45	1664.300	36.984		
TOTAL	49	1715.780			

TABLE 5. PRECISION OF THE CERIODAPHNIA REPRODUCTION TEST, USING NAPCP AS A REFERENCE TOXICANT[a]

Test	NOEC (mg/L)	LOEC (mg/L)	Chronic Value (mg/L)
1[b]	0.25	0.50	0.35
2[c]	0.20	0.60	0.35
3	0.20	0.60	0.35
4[d]	0.30	0.60	0.42
5	0.30	0.60	0.42
6	0.30	0.60	0.42
7	0.30	0.60	0.42
8	0.30	0.60	0.42
9	0.30	0.60	0.42

[a] For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

[b] Concentrations used in Test 1 were; 0.03, 0.06, 0.12, 0.25, 0.50, 1.0 mg NaPCP/L.

[c] Concentrations used in Tests 2 and 3 were, 0.007, 0.022, 0.067, 0.20, 0.60 mg NaPCP/L.

[d] Concentrations used in Tests 4 through 9 were, 0.0375, 0.075, 0.150, 0.30, 0.60 mg NaPCP/L.

SECTION 14

TEST METHOD[1,2]

ALGAL (SELENASTRUM CAPRICORNUTUM) GROWTH TEST
METHOD 1003.0

1. SCOPE AND APPLICATION

1.1 This method measures the chronic toxicity of whole effluents and receiving water to the fresh water alga, *Selenastrum capricornutum*, during a four-day, static exposure. The effects include the synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components which adversely affect the physiological and biochemical functions of the test organisms.

1.2 Detection limits of the toxicity of an effluent or pure substance are organism dependent.

1.3 Single or multiple excursions in acute toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling, and because the test chambers are not sealed, highly volatile and highly degradeable toxicants in the source may not be detected in the test.

1.4 This test is very versatile because it can also be used to identify wastewaters which are biostimulatory and may cause nuisance growths of algae, aquatic weeds, and other organisms at higher trophic levels.

1.5 This method is restricted to use by or under the supervision of professionals experienced in aquatic toxicity testing.

2. SUMMARY OF METHOD

2.1 A *Selenastrum* population is exposed in a static system to a series of concentrations of effluent, or to receiving water, for 96 h. The response of the population is measured in terms of changes in cell density (cell counts per mL), biomass, chlorophyll content, or absorbance. By extending the test to 14 days, it may be used to measure the algal growth potential of wastewaters and surface waters.

3. DEFINITIONS

(Reserved for addition of terms at a later date.)

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[1] The format used for this method was taken from Kopp, 1983.

[2] This method was adapted from Miller et al, 1978.

4. INTERFERENCES

4.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities and Equipment).

4.2 Adverse effects of high concentrations of suspended and/or dissolved solids, and extremes of pH, may mask the presence of toxic substances.

4.3 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling and Sample Handling).

4.4 Pathogenic and/or predatory organisms in the dilution water and effluent may affect test organism survival, and confound test results.

4.5 The amount of natural nutrients in the effluent or dilution water may confound test results.

5. SAFETY

5.1 See Section 3, Safety and Health.

6. APPARATUS AND EQUIPMENT

6.1 Laboratory Selenastrum culture unit -- See culturing methods below. To test effluent toxicity, sufficient numbers of log-phase-growth organisms must be available.

6.2 Samplers -- Automatic sampler capable of collecting a 24-h composite sample of 1 L.

6.3 Sample containers -- for sample shipment and storage see Section 8, Effluent and Receiving Water Sampling and Sample Handling.

6.4 Environmental chamber, incubator, or equivalent facility -- with cool-white fluorescence illumination (60 $uE/m^2/s$, or 400 ± 40 ft-c) and temperature control (24 ± 2^oC).

6.5 Mechanical shaker -- Capable of providing orbital motion at the rate of 100 cycles per minute (cpm)

6.6 Light meter -- with a range of 0-200 $uE/m^2/s$ (0-1000 ft-c).

6.7 Water purification system -- Millipore Super-Q or equivalent.

6.8 Balance -- Analytical, capable of accurately weighing 0.0001 g.

6.9 Reference weights, Class S -- for checking performance of balance.

6.10 Glass or electronic thermometers -- for measuring water temperatures.

6.11 Bulb-thermograph or electronic-chart type thermometers -- for continuously recording temperature.

6.12 National Bureau of Standards Certified thermometer (see EPA Method 170.1, USEPA 1979b).

6.13 Meters: pH and specific conductivity -- for routine physical and chemical measurements. Unless the test is being conducted to specifically measure the effect of one of the above parameters, a portable, field-grade instrument is acceptable.

6.14 Fluorometer (Optional) -- Equipped with chlorophyll detection light source, filters, and photomultiplier tube (Turner Model 110 or equivalent).

6.15 UV-VIS spectrophotometer -- capable of accommodating 1-5 cm cuvettes.

6.16 Cuvettes for spectrophotometer -- 1-5 cm light path.

6.17 Electronic particle counter (Optional) -- Coulter Counter, ZBI, or equivalent, with mean cell (particle) volume determination.

6.18 Microscope -- with 10X, 45X, and 100X objective lenses, 10X ocular lenses, mechanical stage, substage condensor, and light source (inverted or conventional microscope).

6.19 Counting chamber -- Sedgwick-Rafter, Palmer-Maloney, or hemocytometer.

6.20 Centrifuge -- with swing-out buckets having a capacity of 15-100 mL.

6.21 Centrifuge tubes -- 15-100mL, screw-cap.

6.22 Filtering apparatus -- for membrane and/or glass fiber filters.

6.23 Volumetric flasks and graduated cylinders -- Class A, 10-1000 mL, borosilicate glass, for culture work and preparation of test solutions.

6.24 Volumetric pipets-- Class A, 1-100 mL.

6.25 Serological pipets-- 1-10 mL, graduated.

6.26 Pipet bulbs and fillers -- Propipet^R, or equivalent.

6.27 Wash bottles -- for rinsing small glassware, instrument electrodes, and probes.

6.28 Culture flasks -- 1-4 L borosilicate, erlenmeyer flasks.

6.29 Test flasks -- 125 or 250 mL borosilicate, erlenmeyer flasks.

6.30 Preparation of glassware -- prepare all graduated cylinders, test flasks, bottles, volumetric flasks, centrifuge tubes and vials used in algal bioassays as follows:

6.30.1 Wash with non-phosphate detergent solution, preferably heated to 50°C or hotter. Brush the inside of flasks with a stiff-bristle brush to loosen any attached material. The use of a commercial laboratory glassware washer or heavy-duty kitchen dishwasher (under-counter type) is highly recommended.

6.30.2 Rinse thoroughly with tap water, and drain well.

6.30.3 All new test flasks, and all flasks which through use may become contaminated with toxic organic substances, must be rinsed with acetone or heat-treated before use. To thermally degrade organics, place glassware in a high temperature oven at 400°C for 30 min. After cooling, proceed with the next step.

6.30.4 If acetone is used in 6.30.3, rinse thoroughly with tap water. If the heat treatment is used, go directly to 6.30.5.

6.30.5 Carefully rinse with a 10% solution (by volume) of reagent grade hydrochloric acid (HCl); fill vials and centrifuge tubes with the 10% HCl solution and allow to stand a few minutes; fill all larger containers to about one-tenth capacity with HCl solution and swirl so that the entire surface is bathed.

6.30.6 Rinse with tap water and drain well.

6.30.7 To neutralize any residual acid, rinse with a saturated solution of Na_2CO_3.

6.30.8 Rinse five times with tap water and then five times with deionized or distilled water.

6.30.9 Dry in an oven, cover the mouth of each vessel with aluminum foil or other closure, as appropriate, before storing.

6.31 Use of sterile, disposable pipets will eliminate the need for pipet washing and minimize the possibility of contaminating the cultures with toxic substances.

7. REAGENTS AND CONSUMABLE MATERIALS

7.1 Reagent water -- defined as carbon-filtered distilled or deionized water which does not contain substances which are toxic to the test organisms. A water purification system may be used to generate reagent water (see paragraph 6.7 above).

7.2 Effluent, surface water, and dilution water -- see Section 7, Dilution Water, and Section 8, Effluent and Receiving Water Sampling and Sample Handling.

7.3 Reagents for hardness and alkalinity tests (see EPA Methods 130.2 and 310.1, USEPA 1979b).

7.4 pH buffers 4, 7, 8 and 10 (or as per instructions of instrument manufacturer) for standards and calibration check (see USEPA Method 150.1, USEPA 1979b).

7.5 Laboratory quality assurance samples and standards for the above methods.

7.6 Specific conductivity standards (see EPA Method 120.1, USEPA 1979b).

7.7 Reference toxicant solutions (see Section 4, Quality Assurance).

7.8 Acetone -- pesticide quality or equivalent.

7.9 Dilute hydrochloric (or nitric) acid -- carefully add 10 mL of concentrated HCl to 90 mL of reagent water.

7.10 Test Organisms -- log-phase-growth Selenastrum capricornutum. See information on culturing methods below.

7.10.1 Culture Medium

7.10.1.1 The culture medium is used to maintain the stock cultures of the test organisms, for the control flasks in each test, and as a diluent in tests to measure the toxicity of effluents and receiving waters.

7.10.1.2 Prepare five stock nutrient solutions using reagent grade chemicals as described in Table 1.

7.10.1.3 Add 1 mL of each stock solution, in the order listed in Table 1, to approximately 900 mL of distilled or deionized water. Mix well after the addition of each solution. Dilute to 1 L, mix well, and adjust the pH to 7.5 ± 0.1, using 0.1N sodium hydroxide or hydrochloric acid, as appropriate. The final concentration of macronutrients and micronutrients in the culture medium is given in Table 2.

7.10.1.4 Immediately filter the pH-adjusted medium through a 0.45um pore diameter membrane at a vacuum of not more than 380 mm (15 in.) mercury, or at a pressure of not more than one-half atmosphere (8 psi). Wash the filter prior to use by passing 500 mL of distilled water through it.

7.10.1.5 If the filtration is carried out with sterile apparatus, filtered medium can be placed immediately into sterile culture flasks, and no further sterilization steps are required before the inoculation of the medium. The medium can also be sterilized by autoclaving after it is placed in the culture flasks. However, the pH should be checked after autoclaving to determine if it was changed.

TABLE 1. NUTRIENT STOCK SOLUTIONS FOR MAINTAINING ALGAL STOCK CULTURES AND TEST CONTROL CULTURES.

Nutrient Stock Solution	Compound	Amount dissolved in 500 mL Distilled Water
1	$MgCl_2 \cdot 6H_2O$	6.08 g
	$CaCl_2 \cdot 2H_2O$	2.20 g
	H_3BO_3	92.8 mg
	$MnCl_2 \cdot 4H_2O$	208.0 mg
	$ZnCl_2$	1.64 mg[a]
	$FeCl_3 \cdot 6H_2O$	79.9 mg
	$CoCl_2 \cdot 6H_2O$	0.714 mg[b]
	$Na_2MoO_4 \cdot 2H_2O$	3.63 mg[c]
	$CuCl_2 \cdot 2H_2O$	0.006 mg[d]
	$Na_2EDTA \cdot 2H_2O$	150.0 mg
2	$NaNO_3$	12.750 g
3	$MgSO_4 \cdot 7H_2O$	7.350 g
4	K_2HPO_4	0.522 g
5	$NaHCO_3$	7.50 g

[a] $ZnCl_2$ - Weigh out 164 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #1.

[b] $CoCl_2 \cdot 6H_2O$ - Weigh out 71.4 mg and dilute to 100 mL. Add 1 mL of this solution to Stock #1.

[c] $Na_2MoO_4 \cdot 2H_2O$ - Weigh out 36.6 mg and dilute to 10 mL. Add 1 mL of this solution to Stock #1.

[d] $CuCl_2 \cdot 2H_2O$ - Weigh out 60.0 mg and dilute to 1000 mL. Take 1 mL of this solution and dilute to 10 mL. Take 1 mL of the second dilution and add to Stock #1.

Table 2. FINAL CONCENTRATION OF MACRONUTRIENTS AND MICRONUTRIENTS IN THE CULTURE MEDIUM

Macronutrient	Concentration (mg/L)	Element	Concentration (mg/L)
$NaNO_3$	25.5	N	4.20
$MgCl_2 \cdot 6H_2O$	12.2	Mg	2.90
$CaCl_2 \cdot 2H_2O$	4.41	Ca	1.20
$MgSO_4 \cdot 7H_2O$	14.7	S	1.91
K_2HPO_4	1.04	P	0.186
$NaHCO_3$	15.0	Na	11.0
		K	0.469
		C	2.14

Micronutrient	Concentration (ug/L)	Element	Concentration (ug/L)
H_3BO_3	185	B	32.5
$MnCl_2 \cdot 4H_2O$	416	Mn	115
$ZnCl_2$	3.27	Zn	1.57
$CoCl_2 \cdot 6H_2O$	1.43	Co	0.354
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3 \cdot 6H_2O$	160	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300	--	----

7.10.1.6 Unused sterile medium should not be stored in the (250 mL) test culture flasks more than one week prior to use, because there may be substantial loss of water by evaporation.

7.10.2 Algal Cultures

7.10.2.1 Test organisms -- *Selenastrum capricornutum*, a unicellular coccoid green alga. See Section 6, Test Organisms, for information on sources of "starter" cultures.

7.10.2.2 Stock algal cultures

7.10.2.2.1 Upon receipt of the "starter" culture (usually about 10 mL), a stock culture is initiated by aseptically transferring 1 mL to a culture flask containing control algal culture medium (prepared as described above). The volume of stock culture medium initially prepared will depend upon the number of test flasks to be inoculated later from the stock, or other planned uses, and may range from 25 mL in a 125 mL flask to 2 L in a 4-L flask. The remainder of the starter culture can be held in reserve for up to six months in a refrigerator (in the dark) at 4°C.

7.10.2.2.2 Maintain the stock cultures at 24 ± 2°C, under continuous "Cool-White" fluorescent lighting of 86 ± 8.6 uE/m^2/s, or 400 ± 40 ft-c. Shake continuously at 100 cpm or once daily by hand.

7.10.2.2.3 Transfer 1 to 2 mL of stock culture weekly to 1 L of new culture medium to maintain a continuous supply of "healthy" cells for tests. Aseptic techniques should be used in maintaining the algal cultures, and extreme care should be exercised to avoid contamination.

7.10.2.2.4 To maintain unialgal culture material over a long period of time, it is advantageous to use a semi-solid medium containing 1.0% agar. The medium is placed in sterile Petri dishes, and a 1-mL portion of a liquid algal culture is streaked onto it and incubated as described above. Place rubber bands around the petri dishes to reduce evaporation loss of the medium. Fresh (liquid) stock cultures may be started at four week intervals by transfer of cells from a single clone in a petri dish to an appropriate volume of liquid medium.

8. SAMPLE COLLECTION, PRESERVATION AND HANDLING

8.1 See Section 8, Effluent and Receiving Water Sampling and Sample Handling.

9. CALIBRATION AND STANDARDIZATION

9.1 See Section 4, Quality Assurance.

10. QUALITY CONTROL

10.1 See Section 4, Quality Assurance.

11. PROCEDURES

11.1 TEST SOLUTIONS

11.1.1 Surface Waters

11.1.1.1 Surface water toxicity is determined with samples used directly as collected.

11.1.2 Effluents

11.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. One of two dilution factors, approximately 0.3 or 0.5, is commonly used. A dilution factor of approximately 0.3 allows testing between 100% and 1% effluent using only five effluent concentrations (100%, 30%, 10%, 3%, and 1%). This series of dilutions minimizes the level of effort, but because of the wide interval between test concentrations provides poor test precision (± 300%). A dilution factor of 0.5 provides greater precision (± 100%), but requires several additional dilutions to span the same range of effluent concentrations. Improvements in precision decline rapidly as the dilution factor is increased beyond 0.5

11.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 10%, 3%, 1%, 0.3%, and 0.1%). If a high rate of mortality is observed during the first 1 to 2 h of the test, additional dilutions at the lower range of effluent concentrations can be added.

11.1.2.3 The volume of effluent required for the test is 1 L. Prepare enough test solution at each effluent concentration (approximately 700 mL) to provide 100 mL of test solution for each of three replicate test chambers and 400 mL for chemical analyses.

11.1.3 Dilution water may consist of stock culture medium without the EDTA, or other water such as surface water, depending on the objectives of the test. However, if water other than the stock culture medium is used for dilution water, 1 mL of each stock nutrient solution (except for EDTA) should be added per litre of dilution water. Surface waters used as dilution water must be filtered through a 0.45 um pore diameter filter, such as a GF/A, GF/C, or equivalent filter.

11.1.4 Effluents may be toxic and/or nutrient poor. "Poor" growth in an algal toxicity test, therefore, may be due to toxicity or nutrient limitation, or both. To eliminate false negative results due to low nutrient concentrations, 1 mL of each stock nutrient solution (except EDTA) is added per litre of effluent prior to use in preparing the test dilutions. Thus, all test treatments and controls will contain at least the basic amount of nutrients.

11.1.5 If the growth of the algae in the test solutions is to be measured with an electronic particle counter, the effluent and dilution water must be filtered through a GF/A, GF/C, or equivalent pore diameter filter, and checked for "background" particle count before it is used in the test.

11.1.6 If samples contain volatile substances, the test sample should be added below the surface of the dilution water towards the bottom of the test container through an appropriate delivery tube.

11.2 PREPARATION OF INOCULUM

11.2.1 The inoculum is prepared no more than 2 to 3 h prior to the beginning of the test, using *Selenastrum capricornutum* harvested from a four- to seven-day stock culture. Each milliliter of inoculum must contain enough cells to provide an initial cell density of 10,000 cells/mL in the test flasks. Assuming the use of 250 mL flasks, each containing 100 mL of test solution, the inoculum must contain 1,000,000 cells/mL. Estimate the volume of stock culture required to prepare the inoculum as described in the following example:

> If the seven- to 10-day stock culture used as the source of the inoculum has a cell density of 2,000,000 cells/mL, a test employing 25 flasks, each containing 100 mL of test medium and inoculated with a total of 1,000,000 cells, would require 25,000,000 cells or 12.5 mL of stock solution (25,000,000/2,000,000) to provide sufficient inoculum. It is advisable to use a volume 20 to 50% in excess of the minimum volume required, to cover accidental loss in transfer and handling.

1. Determine the density of cells (cells/ml) in the stock culture (for this example, assume 2,000,000 per mL).
2. Calculate the required volume of stock culture as follows:

$$\text{Volume (mL) of Stock Culture Required} = \frac{\text{Number of flasks to be used} \times \text{Volume of Test Solution/Flask} \times 10{,}000\ \text{cells/mL}}{\text{Cell density (cells/mL) in the stock culture}}$$

$$= \frac{25\ \text{flasks} \times 100\ \text{mL/flask} \times 10{,}000\ \text{cells/mL}}{2{,}000{,}000\ \text{cells/mL}}$$

$$= 12.5\ \text{mL Stock Culture}$$

3. Centrifuge 20 mL of stock culture at 1000 x g for 5 min. This volume will provide a 50% excess in the number of cells.
4. Decant the supernatant and resuspend the cells in 15 mL of distilled or deionized water.
5. Repeat the centrifugation and decantation step, and resuspend the cells in 15 mL distilled or deionized water.
6. Mix well and determine the cell density in the makeup water. Some cells will be lost in the concentration process.

Dilute the cell concentrate as needed to obtain a cell density of 1,000,000 cells/mL, and check the cell density in the final inoculum.

11.3 START OF THE TEST

11.3.1 Tests should begin as soon as possible, preferably within 24 h of sample collection. If the persistence of the sample toxicity is not known, the maximum holding time should not exceed 36 h. In no case should the test be started more than 72 h after sample collection. Just prior to testing, the temperature of the sample should be adjusted to that of the test (24 ± 2°C) and maintained at that temperature until portions are added to the dilution water.

11.3.2 The test begins when the algae are added to the test flasks.

1. Mix the inoculum well, and add 1 mL to the test solution in each flask.
2. Make a final check of the cell density in three of the test solutions at time "zero " (within 2 h of the inoculation).

11.4 LIGHT, PHOTOPERIOD, AND TEMPERATURE

11.4.1 Test flasks are incubated under continuous illumination at 86 ± 8.6 uE/m^2/s (400 ± 40 ft-c), at 24 ± 2°C, and should be shaken continously at 100 cpm on a mechanical shaker or twice daily by hand. Flask positions in the incubator should be randomly rotated each day to minimize possible spatial differences in illumination and temperature on growth rate. If it can be verified that test specification are met at all positions, this need not be done.

11.5 ROUTINE CHEMICAL AND PHYSICAL DETERMINATIONS

11.5.1 Measure the pH and specific conductivity of the highest, midrange, and lowest effluent concentrations and the dilution water when the test dilutions are prepared. Additional measurements may be appropriate depending on the test objectives.

11.6 OBSERVATIONS DURING THE TEST

11.6.1 Toxic substances in the test solutions may degrade or volatilize rapidly, and the inhibition in algal growth may be detectable only during the first one to two days in the test. It may be desirable, therefore, to determine the algal growth response daily.

11.7 TERMINATION OF THE TEST

11.7.1 The test is terminated 96 h after initiation. The algal growth in each flask is measured by one of the following methods: (a) cell counts, (b) chlorophyll content, or (3) turbidity (light absorbance). Regardless of the method used to monitor growth, the algae in the test solutions should be checked under the microscope to detect abnormalities in cell size or shape.

11.7.2 Cell counts

11.7.2.1 Automatic Particle Counters

11.7.2.1.1 Several types of automatic electronic and optical particle counters are available for use in the rapid determination of cell density (cells/mL) and mean cell volume (MCV) in um^3/cell. The Coulter Counter is widely used and is discussed in detail by Miller et al., 1978.

11.7.2.1.2 If biomass data are desired for algal growth potential measurements, a Model ZBI or ZB Coulter Counter is used. However, the instrument must be calibrated with a reference sample of cells of known volume.

11.7.2.1.3 When the Coulter Counter is used, an aliquot (usually 1 mL) of the test culture is suspended in a 1% sodium chloride electrolyte (such as Isoton^R), in a ratio of 1 mL of test culture to 9 mL (or 19 mL) of 0.22-um filtered saline solution (dilution of 10:1 or 20:1). The resulting dilution is counted using an aperture tube with a 100-um diameter aperature. Each cell (particle) passing through the aperture causes a voltage drop proportional to its volume. Depending on the model, the instrument stores the information on the number of particles and the volume of each, and calculates the mean cell volume.

The following procedure is used:

1. Mix the algal culture in the flask thoroughly by swirling the contents of the flask approximately six times in a clockwise direction, and then six times in the reverse direction; repeat the two-step process at least once.
2. At the end of the mixing process, stop the motion of the liquid in the flask with a strong brief reverse mixing action, and quickly remove 1 mL of cell culture from the flask with a sterile pipet.
3. Place the aliquot in a counting beaker, and add 9 mL (or 19 mL) of electrolyte solution (such as Coulter ISOTON^R).
4. Determine the cell density (and MCV, if desired).

11.7.2.2 Manual microscope counting methods

11.7.2.2.1 Cell counts may be determined using a Sedgwick-Rafter, Palmer-Maloney, hemocytometer, inverted microscope, or similar methods. For details on microscope counting methods, see APHA 1985 and Weber 1973. Whenever feasible, 400 cells per replicate are counted to obtain ± 10% precision at the 95% confidence level. This method has the advantage of allowing for the direct examination of the condition of the cells.

11.7.3 Chlorophyll Content

11.7.3.1 Chlorophyll may be measured in-vivo fluorometrically, or in-vitro either fluorometrically or spectrophotometrically. In-vivo fluorometric measurements are recommended because of the simplicity and sensitivity of the technique and rapidity with which the measurements can be made (Rehnberg et al., 1982)

The measurements are made as follows:

1. Adjust the "blank" reading of the fluorometer using the filtrate from an equivalent dilution of effluent filtered through a 0.45 um particle retention filter.
2. Mix the contents of the test culture flask by swirling successively in opposite directions (at least three times), and remove 1 mL of culture from the flask with a sterile pipet.
3. Place the aliquot in a small disposable vial and record the fluorescence as soon as the reading stabilizes. (Do not allow the sample to stand in the instrument more than 1 min.)
4. Discard the sample.

11.7.4 Turbidity (Absorbance)

11.7.4.1 A second rapid technique for growth measurement involves the use of a spectrophotometer to determine the turbidity, or absorbance, of the cultures at a wavelength of 750 nm. Because absorbance is a complex function of the volume, size, and pigmentation of the algae, it would be useful to construct a calibration curve to establish the relationship between absorbance and cell density.

The algal growth measurements are made as follows:

1. A blank is prepared as described for the fluorometric analysis.
2. The culture is thoroughly mixed as described above.
3. Sufficient sample is withdrawn from the test flask with a sterile pipet and transferred to a 1- to 5-cm cuvette.
4. The absorbance is read at 750 nm and divided by the light path length of the cuvette, to obtain an "absorbance-per-centimeter" value.
5. The 1-cm absorbance values are used in the same manner as the cell counts.

11.7.5 Biomass

11.7.5.1 The results of algal growth potential tests are commonly expressed in terms of biomass (mg dry wgt organic matter/L). Algal biomass can be calculated from cell counts and mean cell volumes, or can be measured directly by gravimetric methods.

11.7.5.2. Biomass calculated from Cell Counts and Mean Cell Volumes

Algal biomass is calculated as follows:

Dry Weight (mg/L) = Cells/L X MCV(um^3) X mg organic matter/um^3

Where: MCV = either:

1. The "measured" or "actual" cell volume, such as determined with a Coulter Counter or determined by microscopic examination, or

2. When actual measurements are not available, a value of 60 um^3 is used as an estimate of the MCV. A standard value of 3.6 X 10^{-10}mg is used for the weight of organic matter/um^3.

If the cell count is 1,000,000 cells/mL:

$$\text{Biomass (mg/L)} = 1{,}000{,}000 \text{ cells/mL} \times 1000 \text{ mL/L} \times 60 \text{ um}^3\text{/cell} \times$$

$$3.6 \times 10^{-10} \text{ mg/um}^3$$

$$= 21.6 \text{ mg/L}$$

11.7.5.3 Biomass by Direct, Gravimetric, Dry Weight Measurements

11.7.5.3.1 Direct, gravimetric methods of measuring biomass are appropriate where the harvestable biomass per test flask is greater than 10 mg dry weight. This condition is met where the cell density is equal to or exceeds 5,000,000 cells/mL. This cell density would not ordinarily be achieved in a 96-h test, but would be applicable with a 14-day test.

11.7.5.3.2 If the cell density is large enough to warrant use of the gravimetric method, proceed as follows, treating each flask separately:

1. Centrifuge the entire contents of each flask at 1000 X g for 5 min.
2. Decant the supernatant (do not retain more than 10 mL of culture medium with the cells). Note: Caution must be exercised to avoid disturbing the sedimented cells when decanting the supernatant.
3. Resuspend the cells in 10 mL distilled water.
4. Centrifuge and decant, as in (2) above.
5. Transfer the sedimented cells from each flask to a separate weighed crucible or weighing pan.
6. Dry overnight at 70°C.
7. Cool in a desiccator and weigh to the nearest 0.1 mg.
8. Report the dry weight in mg/L.

11.7.5.3.3 The cells may also be concentrated and dried on 0.45-um pore diameter membrane filters or on equivalent glass fiber or other filters, as follows:

1. Dry the filters for 2 h at 70°C (temperatures above 75°C may cause the pores to close in membrane filters.
2. Allow the filters to cool in a desiccator for at least 1 h before weighing.
3. Filter the contents of the test flask using a vacuum of 380 mm of mercury, or a pressure not to exceed one-half atmosphere.
4. Rinse the filter funnel with 50 mL of distilled water using a wash bottle, and allow the rinsings to pass through the filter. The rinse serves to transfer all the algae to the filter and washes the nutrient salts through the filter.
5. Dry the filters overnight at 70°C (to constant weight), cool in a desiccator for 1 h, and weigh to the nearest 0.1 mg.

11.8 SUMMARY OF TEST CONDITONS

11.8.1 A summary of test conditions is listed in Table 3.

11.9 ACCEPTABILITY OF TEST RESULTS

11.9.1 The test results are acceptable if the algal cell density in the control flasks exceeds 10^6cells/mL at the end of the test, and does not vary more than 10% among replicates.

12. CALCULATIONS

12.1 TOXICITY DATA

12.1.1 The data can be analyzed by Dunnett's Procedure or Probit Analysis. The results of the toxicity test are expressed as an NOEC and/or EC1 (threshold effect concentration). The chronic value can also be determined if desired.

12.1.2 The NOEC and LOEC (MATC), and chronic value are based on comparisons of the growth of the control organisms with each of the toxicant concentrations, using Dunnett's Test.

12.1.3 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. Tests for normality and homogeneity of variance are included in the Appendix. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

12.1.3 Dunnett's Procedure:

12.1.3.1 Dunnett's Procedure (Dunnett, 1955) includes an analysis of variance (ANOVA) followed by a comparison of each toxicant concentration mean with the control mean. The error value calculated in the ANOVA is used in the comparison of the control and treatment means. The computer program listed in the Appendix generates output which includes an ANOVA table, a statement about each treatment mean that can be used to identity the NOEC and LOEC, and the minimum difference between treatment and control means that can be detected as statistically significant. Dunnett's Procedure is used when the assumptions of normality and homogeneity of variance are met. If they are not met, Steel's Many-One Rank Test is used.

12.1.3.2 The sample set of cell counts shown in Table 4 were transformed to $\log_{10}$ to help meet the assumptions for normality and homogeneity of variance. The logs were input to the Dunnett's program listed in the Appendix.

12.1.3.3. The results of the analysis of variance of the log transformed data from Table 4 are shown in Table 5, and indicate a statistically significant difference in survival among cadmium concentrations. The computer output for the comparison of treatment means with the control mean is shown below:

```
THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 2
(5 UG CD/L) AND CONTROL.

THERE IS A SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 3
(10 UG CD/L) AND CONTROL.

THERE IS A SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 4
(20 UG CD/L) AND CONTROL.

THERE IS A SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 5
(40 UG CD/L) AND CONTROL.

THERE IS A SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 6
(80 UG CD/L) AND CONTROL.
```

12.1.3.4 The NOEC and LOEC, as determined from the Dunnett computer analysis, were 5 ug Cd/L and 10 ug Cd/L, respectively. (Note that the Dunnett program considers the control as Concentration 1, so that Concentration 2 in the output is 5 ug Cd/L in the test). The MATC was 5 - 10 ug Cd/L

12.1.3.5 For this set of data, the minimum difference between the control mean and a treatment mean that can be detected as statistically significant is 225,339 cells/mL. This represents a 18.1% reduction in the response from the control.

12.1.3.6 The chronic value is determined by calculating the geometric mean (GM) of the NOEC and LOEC, as follows:

$\text{Log}_{10}\ \text{NOEC} = \text{Log}_{10}\ 5.0 = 0.69897$

$\text{Log}_{10}\ \text{LOEC} = \text{Log}_{10}\ 10.0 = 1.00000$

$\text{ChV} = \text{Antilog}\ (0.69897 + 1.0000)/2 = \text{Antilog}\ 0.8495$

$\text{ChV} = 7.07$ ug Cd/L

12.1.4 Probit Analysis

12.1.4.1 Although the assumptions for Probit Analysis are not met in the classical sense because of the very nature of the algal growth data, the analysis is used to obtain an estimate of the EC1. To carry out the Probit Analysis, use the growth response data, such as cell counts, in-vivo chlorophyll fluorescence, or absorbance, for each set of three replicate flasks in a given treatment. The special Probit program listed in the Appendix uses the algal count data, determines the mean, and converts it to an "inhibition proportion" using the following formula:

$$I = \frac{C - T}{C}$$

Where: C = The mean growth in the control flasks
T = The mean growth at a given effluent concentration.

12.1.4.2 The cell counts were entered into the "Algal" Probit program listed in the Appendix. The EC1 was found to be 2.27 ug Cd/L, with upper and lower confidence limits of 3.42 and 1.20 ug Cd/L, respectively, at $P = 0.05$. An example of how the data are entered in the program and the program output are provided in the Appendix.

12.2 BIOSTIMULATION

12.2.1 Where the growth response in effluent (or surface water) exceeds growth in the control flasks, the percent stimulation, S(%), is calculated as shown below. Values which are significantly greater than the control indicate a possible degrading enrichment effect on the receiving water (Walsh, et al., 1980b):

$$S(\%) = \frac{T - C}{C} \times 100$$

13. TEST PRECISION AND ACCURACY

13.1 PRECISION

13.1.1 Data from repetitive 96-h toxicity tests, conducted with three reference toxicants, are shown in Table 6. The relative standard deviation (coefficient of variation) of the LC1s ranged from 47% to 83%.

13.2 ACCURACY

13.2.1 The accuracy of toxicity tests can not be determined.

TABLE 3. SUMMARY OF RECOMMENDED TEST CONDITIONS FOR THE ALGAL GROWTH TEST

1. Test type:	Static
2. Temperature:	$24 \pm 2^{o}C$
3. Light quality:	"Cool white" fluorescent lighting
4. Light intensity:	86 ± 8.6 uE/m^{2}/s (400 ± 40 ft-c)
5. Photoperiod:	Continuous illumination
6. Test flask size:	125 mL or 250 mL
7. Test solution volume:	50 mL or 100 mL
8. Age of stock culture used for inoculum:	4 to 7 days
9. Initial cell density:	10,000 cells/mL
10. Number of replicates per concentration:	3
11. Shaking rate:	100 cpm continuous, or twice daily by hand
12. Dilution water:	Algal stock culture medium without EDTA, or surface water
13. Dilution factor:	Approximately 0.3 or 0.5
14. Test duration:	96 h
15. Effect measured:	Growth (cell counts, chlorophyll fluorescence, absorbance, biomass)
16. End point(s):	EC1, NOEC, S(%)

TABLE 4. SAMPLE DATA FROM ALGAL TOXICITY TEST WITH CADMIUM CHLORIDE

Toxicant Concentration (ug Cd/L)	Growth Response: Cells/mL / 1000	Cells/mL / 1000 (Log_{10})	Percent Inhibition of Growth I(%)
0 (Control)	1209	3.082	
	1180	3.072	0
	1340	3.127	
5	1212	3.084	
	1186	3.074	3.4
	1204	3.081	
10	826	2.917	
	628	2.798	39.1
	816	2.912	
20	493	2.693	
	416	2.619	64.5
	413	2.616	
40	127	2.104	
	147	2.167	88.7
	147	2.167	
80	49.3	1.693	
	40.0	1.602	96.4
	44.0	1.643	

TABLE 5. ANALYSIS OF VARIANCE OF CELL COUNT DATA IN TABLE 4 TRANSFORMED TO LOG_{10}.

Source	DF	Sum of Squares	Mean Square	Calculated F	Tabular $F_{0.05}$
Among	5	4.995	0.999	560.183[a]	3.11
Within	12	0.021	0.002		
Total	17	5.016			

[a] Significant at P = 0.05.

TABLE 6. PRECISION OF THE SELENASTRUM CAPRICORNUTUM, 96-H TOXICITY TEST, USING REFERENCE TOXICANTS

Test No.	Toxicant											
	Cadmium Chloride				Sodium Pentachlorophenate				Sodium Dodecyl Sulfate			
	LC1[a]			NOEC[b]	LC1			NOEC	LC1			NOEC
	(ug Cd/L)	UL	LL	(ug Cd/L)	(ug/L)	UL	LL	(ug/L)	(mg/L)	UL	LL	(mg/L)
1	0.201	0.272	0.181	LT 0.49[c]	20.7	27.6	13.9	62.5	2.57	3.13	1.97	5.0
2	0.647	1.33	0.198	LT 10.0	NC	---	---	80.0	1.32	1.77	0.890	2.5
3	.372	5.45	0.220	1.0	16.7	21.6	11.9	40.0	5.57	6.60	4.30	10.0
4	.242	0.446	0.0981	LT 2.0	41.3	46.8	34.8	66.0	6.41	7.52	4.98	7.5
5	.638	0.972	0.352	LT 2.0	40.2	45.5	33.9	LT 66.0	1.26	1.81	0.766	LT 5.0
6	2.37	3.27	1.54	LT 8.0	47.0	53.1	39.8	82.0	2.85	2.98	2.72	5.0
7	2.27	2.98	1.59	5.0	43.4	48.7	37.1	LT 66.0				
8	1.23	1.78	0.748	5.0	84.3	90.0	76.3	102				
9	0.347	0.652	0.137	LT 5.0	40.5	48.4	30.8	LT 66.0				
10	.608	1.01	0.296	LT 5.0	33.4	40.6	25.5	82.0				
11	1.72	2.38	1.11	5.0								
N	11				9				6			
Mean	0.968				40.8				3.33			
SD	0.806				19.3				1.98			
CV	83%				47%				60%			

[a] LC1 (threshold concentration) and upper (UL) and lower (LL) confidence limits determined by Probit Analysis.

[b] NOEC determined with Dunnett's Test.

[c] LT = NOEC less than the lowest concentration tested.

Reference toxicant concentrations used in the toxicity tests are listed below:

Cadmium Chloride (ug Cd/L) :
1: 0.49, 0.95, 1.88, 3.77, 7.27
2: 10.0, 20.0, 40.0, 80.0
3: 1.0, 2.0, 4.0, 8.0, 16.0
4: 2.0, 4.0, 8.0, 16.0, 32.0
5: 2.0, 4.0, 8.0, 16.0, 32.0
6: 8.0, 16.0, 32.0, 64.0, 128
7: 5.0, 10.0, 20.0, 40.0, 80.0
8: 5.0, 10.0, 20.0, 40.0, 80.0
9: 5.0, 10.0, 20.0, 40.0, 80.0
10: 5.0, 10.0, 20.0, 40.0, 80.0
11: 5.0, 10.0, 20.0, 40.0, 80.0

Sodium Pentachlorophenate (ug/L):
1: 62.5, 125, 250, 500, 1000
2: 40.0, 80.0, 160, 320, 640
3: 40.0, 80.0, 160, 320, 640
4: 66.0, 82.0, 102, 128, 160
5: 66.0, 82.0, 102, 128, 160
6: 66.0, 82.0, 102, 128, 160
7: 66.0, 82.0, 102, 128, 160
8: 66.0, 82.0, 102, 128, 160
9: 66.0, 82.0, 102, 128, 160
10: 66.0, 82.0, 102, 128, 320

Sodium Dodecyl Sulfate (mg/L):
1: 2.5, 5.0, 7.5, 10.0, 12.5, 15.0
2: 2.5, 5.0, 10.0, 12.5, 15.0, 20
3: 2.5, 10.0, 12.5, 15.0, 20.0
4: 5.0, 7.5, 12.5, 15.0, 20.0
5: 5.0, 12.5, 20.0, 40.0, 80.0
6: 2.5, 5.0, 12.5, 15.0, 20.0, 40.0

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APPENDIX

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APPENDIX A

VALIDATING NORMALITY AND HOMOGENEITY OF VARIANCE ASSUMPTIONS[1]

1. INTRODUCTION

1.1 Dunnett's Procedure is a parametric procedure and is based on the assumptions that the observations are independent and normally distributed, and that the variance of the observations is homogeneous across all toxicant concentrations and the control. These assumptions should be checked prior to using Dunnett's Procedure, to determine if they have been met. Tests for validating the assumptions are provided in the following discussion. If the tests fail (if the data do not meet the assumptions), a non-parametric procedure such as Steel's Many-One Rank Test may be more appropriate. However, the decision on whether to use parametric or non-parametric tests may be a judgement call, and a statistician should be consulted in selecting the analysis.

2. TEST FOR NORMAL DISTRIBUTION OF DATA

2.1 A formal test for normality is the Chi-Square Goodness of Fit Test. This test compares the observed sample distribution with a normal distribution. An example of the test is provided below.

2.2 The example uses mortality data from the fathead minnow larval survival and growth test with sodium pentachlorophenate (NaPCP) listed in Section 10, and is the same data used in the discussion of the homogeneity of variance determination in Appendix A.3 and the Dunnett's Procedure in Appendix C. The data used in the Dunnett's Procedure has been arc sine square root transformed, and it is the transformed data which will be tested for normality. The transformed data and the mean and standard deviation of the observations at each toxicant concentration, including the control, are listed in Table A.1.

2.3 The first step of the Chi-Square Goodness of Fit Test is to standardize the observations by subtracting the mean from each observation and dividing the difference by the standard deviation. The standardized observations are listed in Table A.2.

2.4 Form five cells as follows: < -1.5; -1.5 to < -0.50; -0.50 to 0.50; > 0.50 to 1.5; > 1.5. Tabulate the number of standardized observations which fall into each of the five cells. These are the observed frequencies, f_i. The expected frequency, F_i, is found by multiplying the area under the standard normal curve over the ith cell limits by the total number of standardized observations, N.

---

[1] Prepared by John Menkedick and Florence Kessler, Computer Sciences Corporation, 26 W. St. Clair St., Cincinnati, Ohio 45268; Phone 513-568-7968.

TABLE A.1. ARC SINE SQUARE ROOT TRANSFORMED DATA FOR CHI-SQUARE TEST

		NaPCP Concentration (ug/L)				
Replicate	Control	3	5	7	11	16
1	1.412	1.107	1.249	1.249	0.991	0.685
2	1.412	1.107	1.412	1.249	1.249	0.580
3	1.249	1.412	1.412	1.107	1.412	0.685
4	1.249	1.107	1.412	1.412	0.785	0.464
Total	5.322	4.733	5.485	5.071	4.437	2.414
Mean	1.330	1.183	1.371	1.254	1.109	0.604
S_i	0.0941	0.1524	0.0815	0.1246	0.2769	0.1054
i	1	2	3	4	5	6

TABLE A.2. EXAMPLE OF CHI-SQUARE TEST FOR NORMALITY:
STANDARDIZED OBSERVATIONS

	NaPCP Concentration (ug/L)					
Replicate	Control	3	5	7	11	16
1	0.8714	- 0.4987	- 1.4969	- 0.0401	- 0.4261	0.7685
2	0.8714	- 0.4987	0.5031	- 0.0401	0.5056	- 0.2277
3	- 0.8608	1.5026	0.5031	- 1.1798	1.0942	0.7685
4	- 0.8608	- 0.4987	0.5031	1.2680	- 1.1701	- 1.3283

2.5 For this example, N = 24. The areas for each cell, the observed frequencies, and the expected frequencies are given in Table A.3.

TABLE A.3. EXAMPLE OF CHI-SQUARE TEST FOR NORMALITY: ACTUAL AND EXPECTED FREQUENCIES

Cell Interval	< -1.5	-1.5 to < -0.5	-0.5 to 0.5	> 0.5 to 1.5	> 1.5
i	1	2	3	4	5
Area	0.067	0.242	0.382	0.242	0.067
F_i	1.608	5.808	9.168	5.808	1.608
f_i	0	6	7	10	1

2.6 The Chi-Square Goodness of Fit Test statistic (X^2) is calculated as follows:

$$X^2 = \sum_{i=1}^{5} \frac{(f_i - F_i)^2}{F_i}$$

2.7 For the data in the example:

$$X^2 = \frac{(0 - 1.608)^2}{1.608} + \frac{(6 - 5.808)^2}{5.808} + \frac{(7 - 9.168)^2}{9.168} + \frac{(10 - 5.808)^2}{5.808} + \frac{(1 - 1.608)^2}{1.608}$$

$$= 5.3825$$

2.8 The decision rule for this test is to compare the critical value, X^2, with four degrees of freedom (No. of cells - 1) and an alpha of 0.01, to the computed X^2. If the computed value exceeds the critical value, conclude that the data are not normally distributed. For this example, the critical value is 13.28. The calculated value, 5.3825, does not exceed the critical value. Thus, the conclusion of the test is that the data are normally distributed.

2.9 In general, if the data fail the test for normality, a transformation such as to log values may normalize the data. After transforming the data, repeat the Chi-Square Goodness of Fit Test for normality.

3. TEST FOR HOMOGENEITY OF VARIANCE

3.1 For Dunnett's Procedure, the variances of the data obtained from each toxicant concentration and the control are assumed to be equal. Bartlett's Test is a formal test of this assumption. In using this test, it is assumed that the data are normally distributed. An example of how this test is performed is provided below:

3.2 The data used in this example are mortality data from a fathead minnow larval survival and growth test, and are the same data used in Appendix C. Since Dunnett's Procedure is performed with arc sine transformed data, Bartlett's Test is performed with the same transformed data. These data are listed in Table A.4, together with the calculated variance for the control and each toxicant concentration.

3.3 The test statistic for Bartlett's Test (Snedecor and Cochran, 1980) is as follows:

$$B = \frac{v[a(\ln \overline{S}^2) - \sum_i \ln S_i^2]}{C}$$

Where: v = Degrees of freedom for each toxicant concentration and control

a = Number of levels of toxicant concentration including the control

$\overline{S}^2$ = The average of the individual variances

C = $1 + [(a+1)/3av]$.

ln = Log_e

3.4 For the data in this example, $v = 3$, $a = 6$, $\overline{S}^2 = 0.0236$, and $C = 1.1296$. The calculated B value is:

$$B = \frac{3\,[6(\ln 0.0236) - \sum_i \ln S_i^2]}{1.1296}$$

$$= \frac{3[6(-3.7465) - (-24.7529)]}{1.1296}$$

$$= 6.0390$$

3.5 Since B is approximately distributed as Chi Square with a - 1 degrees of freedom when the variances are equal, the appropriate critical value for the test is 15.09 for a significance level of 0.01. Since B < 15.09, the conclusion is that the variances are equal.

TABLE A.4 DATA USED IN BARTLETT'S TEST FOR HOMOGENEITY OF VARIANCE

		NaPCP Concentration (ug/L)				
Replicate	Control	3	5	7	11	16
1	1.412	1.107	1.249	1.249	0.991	0.685
2	1.412	1.107	1.412	1.249	1.249	0.580
3	1.249	1.412	1.412	1.107	1.412	0.685
4	1.249	1.107	1.412	1.412	0.785	0.464
Total	5.322	4.733	5.485	5.071	4.437	2.414
Mean	1.330	1.183	1.371	1.254	1.109	0.604
$S_i{}^2$	0.0088	0.0232	0.0066	0.0155	0.0767	0.0111
i	1	2	3	4	5	6

APPENDIX B

ARC SINE SQUARE ROOT TRANSFORMATION[1]

1. Arc Sine transformation consists of determining the angle (in radians) represented by a sine value. In the case of arc sine square root transformation of mortality data, the proportion of dead (or affected) organisms is taken as the sine value, the square root of the sine value is determined, and the angle (in radians) for the square root of the sine value is determined. Whenever the proportion dead is 0 or 1, a special modification of the arc sine square root transformation must be used (Bartlett, 1937). An explanation of the arc sine square root transformation and the modification is provided below.

2. Calculate the response proportion (RP) at each effluent concentration, where:

   RP = (number of dead or "affected" organisms)/(initial number).

   Example: If 8 of 20 animals in a given treatment die:

   $$\begin{aligned} RP &= 8/20 \\ &= 0.40 \end{aligned}$$

3. Transform each RP to Arc Sine, as follows:

   a. For RP = > 0 to < 1:

      $\text{Angle (radians)} = \text{Arc Sine } (RP)^{0.5}$

      Example: If RP = 0.40,

      $$\begin{aligned} \text{Angle} &= \text{Arc Sine } (0.40)^{0.5} \\ &= \text{Arc Sine } 0.6325 \\ &= 0.6847 \text{ radians} \end{aligned}$$

---

[1] From Peltier and Weber (1985).

b. For RP = 0 (no mortality), the following adjustment is made:

Angle (in radians) = Arc Sine $(1/4N)^{0.5}$
Where: N = Number of animals/treatment

Example: If 20 animals are used,

Angle = Arc Sine $(1/80)^{0.5}$
= Arc Sine 0.1118
= 0.1120 radians

c. For RP = 1.0 (100% mortality):

Angle = 1.5708 radians - (radians for RP = 0)

Example: Using above value

Angle = 1.5708 - 0.1120
= 1.4588 radians

APPENDIX C

DUNNETT'S PROCEDURE

1. MANUAL CALCULATIONS[1]

1.1 Dunnett's Procedure is used to compare each concentration mean with the control mean to decide if any of the concentrations differ from the control. This test has an overall error rate of alpha, which accounts for the multiple comparisons with the control. It is based on the assumptions that the observations are independent and normally distributed and that the variance of the observations is homogeneous across all concentration and control. (See Appendix A for a discussion on validating the assumptions). Dunnett's Procedure uses a pooled estimate of the variance, which is equal to the error value calculated in an analysis of variance.

1.2 The data for this example are mortality data from a fathead minnow larval survival and growth test. Since the data are expressed in proportions, a transformation of the square root of the proportion to an arc sine value is required (See Appendix B.) A table of the raw data (Table C.1) and transformed data (Table C.2) are provided below.

TABLE C.1. PROPORTIONS OF SURVIVING TEST ORGANISMS.

Concentration (ug NaPCP/L)	i	Proportion Surviving in Replicate Chambers			
		A	B	C	D
Control	1	1.00	1.00	0.90	0.90
3	2	0.80	0.80	1.00	0.80
5	3	0.90	1.00	1.00	1.00
7	4	0.90	0.90	0.80	1.00
11	5	0.70	0.90	1.00	0.50
16	6	0.40	0.30	0.40	0.20

---

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TABLE C.2. ARC SINE TRANSFORMED DATA FOR DUNNETT'S PROCEDURE

NaPCP (ug/L)	Conc (i)	Transformed Data (Arc Sine in Radians)) Replicate				Total T_i	Mean Y_i
		A	B	C	D		
Control	1	1.412	1.412	1.249	1.249	5.322	1.330
3	2	1.107	1.107	1.412	1.107	4.733	1.183
5	3	1.249	1.412	1.412	1.412	5.485	1.371
7	4	1.249	1.249	1.107	1.412	5.017	1.254
11	5	0.991	1.249	1.412	0.785	4.437	1.109
16	6	0.685	0.580	0.685	0.464	2.414	0.604

1.3 One way to obtain an estimate of the pooled variance is to construct an ANOVA table including all sums of squares, using the following formulas:

Total Sum of Squares: $TSS = \sum_{ij} Y_{ij}^2 - G^2/N$

Between Sum of Squares: $SSB = \sum_{i} T_i^2/n_i - G^2/N$

Within Sum of Squares: $SSW = TSS - SSB$

Where: G = The grand total of all sample observations; $G = \sum_i T_i$

N = The total sample size; $N = \sum_i n_i$

n_i = The number of replicates for concentration "i".

T_i = The total of the replicate measurements for concentration "i".

Y_{ij} = The jth observation for concentration "i".

1.4 Calculations:

Total Sum of Squares: $TSS = \sum_{ij} Y_{ij}^2 - G^2/N$

$= 33.302 - (27.408)^2/24$

$= 2.002$

Between Sum of Squares: $SSB = \sum_i T_i^2/n_i - G^2/N$

$$= 32.875 - (27.408)^2/24$$
$$= 1.575$$

Within Sum of Squares: $SSW = TSS - SSB$

$$= 2.002 - 1.575$$
$$= 0.427$$

1.5 Prepare the ANOVA table as follows:

Source	DF	Sum of Squares(SS)	Mean Square(MS) (SS/DF)	F
Between	$b^* - 1$	SSB	$S_B^2 = SSB/(b-1)$	S_B^2/S_W^2
Within	$N - b$	SSW	$S_W^2 = SSW/(N-b)$	
Total	$N - 1$	TSS		

*Where b = Number of different concentrations, including the control.

1.6 The completed ANOVA table for this data is provided below:

TABLE C.3. COMPLETED ANOVA TABLE FOR DUNNETT'S PROCEDURE

Source	DF	SS	MS	F
Between	6 - 1 = 5	1.575	0.315	13.125
Within	24 - 6 = 18	0.427	0.024	
Total	23	2.002		

1.7 To perform the individual comparisons, calculate the t statistic for each concentration and control combination, as follows:

$$t_i = \frac{(\overline{Y}_1 - \overline{Y}_i)}{[S_w \sqrt{(1/n_1) + (1/n_i)}]}$$

Where: $\overline{Y}_i$ = Mean for each concentration
$\overline{Y}_1$ = Mean for the control
S_w = Square root of the within mean square
n_1 = Number of replicates in the control.
n_i = Number of replicates for concentration "i".

1.8 Table C.4 includes the calculated t values for each concentration and control combination.

TABLE C.4. CALCULATED T VALUES.

Concentration (ug NaPCP/L)	i	t_i
3	2	1.342
5	3	- 0.374
7	4	0.694
11	5	2.108
16	6	6.630

1.9 Since the purpose of the test is only to detect a decrease in survival from the control, a one-sided test is appropriate. The critical value for the one-sided comparison, with an overall alpha level of 0.05, 18 error degrees of freedom, and 5 concentrations excluding the control, is 2.41 (See Table C.5). Comparing each of the calculated t values in Table C.4 with the critical value, the 16 ug NaPCP/L concentration is found to have significantly lower survival than the control (6.630 > 2.41). Thus the NOEC is 11 ug NaPCP/L.

1.10 To quantify the sensitivity of the test, the minimum significant difference (MSD) may be calculated. The formula is as follows:

$$MSD = d\ S_w \sqrt{(1/n_1) + (1/n)}$$

Where: d = Critical value for the Dunnett's Procedure
S_w = The square root of the within mean square
n = The number of replicates at each concentration, assuming an equal number of replicates at all treatment concentrations
n_1 Number of replicates in the control

For example:

$$\begin{aligned} MSD &= 2.41\ (0.155)[\sqrt{(1/4) + (1/4)}] = 2.41\ (0.155)(\sqrt{2/4}) \\ &= 2.41\ (0.155)(0.707) \\ &= 0.264 \end{aligned}$$

1.11 The MSD (0.264) is in transformed units. To determine the MSD in terms of percent survival, carry out the following conversion:

1.11.1. Subtract the MSD from the transformed control mean.

$$1.330 - 0.264 = 1.066$$

1.11.2. Obtain untransformed values for the control mean (1.3305) and the difference (1.0665) obtained in Step 1.

$$[\text{Sine } (1.330)]^2 = 0.943$$
$$[\text{Sine } (1.066)]^2 = 0.766$$

1.11.3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values obtained in Step 2.

$$MSD_u = 0.943 - 0.766 = 0.177$$

1.11.4 For this set of data, the minimum difference between the control mean and a concentration mean that can be detected as statistically significant is 0.177. This represents a decrease in survival of 19% from the control.

2. COMPUTER CALCULATIONS

2.1 This computer program incorporates two analyses: an analysis of variance (ANOVA), and a multiple comparison of treatment means with the control mean (Dunnett's Test). The ANOVA indicates whether there is a significant difference between the results obtained at different toxicant concentrations. The Dunnett Test indicates which toxicant concentration means (if any) are statistically different from the control mean. The program also gives the minimum difference between the control and treatment means that could be detected as statistically significant. The multiple comparison is based on Dunnett, C. W., 1955, "Multiple Comparison Procedure for Comparing Several Treatments with a Control," J. Amer. Statist. Assoc. 50:1096-1121. Tables for one-tailed "F" and Dunnett's "t" (P = 0.05) are included in the program, but are limited to values for eight concentrations, including the control, and six replicates per concentration. If the test design exceeds these limits, it is necessary to consult the "t" and "F" tables listed following the program.

2.2 The program was written in FORTRAN PLUS IV v.3, by James Dryer, and runs on the Digital Equipment Corporation, PDP 11/70 computer under IAS version 3.1. Machine readable copies of the program, and assistance in running it, can be obtained by contacting James Dryer, EMSL-Cincinnati Newtown Facility (FTS 778-8350; comm'l 513-527-8350).

2.3 Description of Data Input and Output from the Computer Program

2.3.1 Data Input

2.3.1.1 Cell growth data from a *Selenastrum* toxicity test, listed in Table 4, Section 14, were used to illustrate the data input process and the output provided by the computer program. As shown below, the program prompts the operator for the following information:

1. The type of transformation to be used with the data.
2. The number of replicates at each concentration.
3. The values (cell growth) obtained from each replicate toxicant concentration.

2.3.1.2 Several transformations are available in the program. Transformation option "3", conversion to $\log_{10}$, was used in the example provided. Six concentrations, each with three replicates, were used in the test. The control is considered as Concentration 1.

2.3.2 Program Output

2.3.2.1 The output from the analyses includes:

1. A table of the original data (cell growth) input to the program.
2. An ANOVA table listing the calculated "F" and tabular "F" values.
3. A series of statements regarding the results of each sequential comparison of a toxicant concentration mean with the control mean.

2.3.2.2 The calculated F from the ANOVA is compared with the tabular F value where P = 0.05, and DF = 5,12. The calculated F (560.183) is larger than the tabular F (3.11), indicating that the survival is significantly different at the different toxicant concentrations.

2.3.2.3 The output from the Dunnett sequential comparisons indicates that there is a significant difference between Concentration 3 and the control (Concentration 1). On the basis of these results, the NOEC is Concentration 2.

2.3.3 Computer Generated Listings of Input and Output from the Computer Program for Dunnett's Test and Analysis of Variance

---

```
   D U N N E T T S   T E S T

 IF YOU NEED INSTRUCTIONS,TYPE 1. OTHERWISE,TYPE 0 - 1

 ANOVA-WITH DUNNETTS COMPARISON OF EACH CONCENTRATION MEAN
 VS THE CONTROL MEAN.


  A ONE-SIDED 'T' AT 95% IS USED IN ALL CASES.


  THE FOLLOWING TRANSFORMATIONS ARE AVAILABLE TO USE ON
  THE VALUES:

  NUMBER      TRANSFORMATION
    0         NONE
    1         SQUARE ROOT(X)
    3         LOG 10(X)
    5        (ARCSIN(SQ. RT(X)/(N+1)+
              ARCSIN(SQ. RT(X+1)/(N+1)))/2
    6         ARCSIN((X)**0.5)


TRANSFORMATION NUMBER = 3
AVERAGE NUMBER OF TEST ORGANISMS AT  BEGINNING PER CONCENTRATION = 100.
INCLUDING CONTROL, NUMBER OF CONCENTRATIONS = 6
NOTE: BEGIN WITH CONTROL(CONCENTRATION NUMBER 1) AND ENTER  IN ASCENDING ORDER.

NUMBER OF REPLICATES IN CONCENTRATION NUMBER   1
= 3
NUMBER OF REPLICATES IN CONCENTRATION NUMBER   2
= 3
NUMBER OF REPLICATES IN CONCENTRATION NUMBER   3
= 3
NUMBER OF REPLICATES IN CONCENTRATION NUMBER   4
= 3
NUMBER OF REPLICATES IN CONCENTRATION NUMBER   5
= 3
NUMBER OF REPLICATES IN CONCENTRATION NUMBER   6
= 3
```

2.3.3 Computer Generated Listings of Input and Output from the Computer Program for Dunnett's Procedure (Continued)

```
 ENTER TEST DATA FROM INDIVIDUAL REPLICATES.

 NOTE: DECIMAL POINT MUST BE USED IN "VALUE".

"VALUE" OF CONCENTRATION NUMBER  1 =

1209.
"VALUE" OF CONCENTRATION NUMBER  1 =

1180.
"VALUE" OF CONCENTRATION NUMBER  1 =

1340.
"VALUE" OF CONCENTRATION NUMBER  2 =

1212.
"VALUE" OF CONCENTRATION NUMBER  2 =

1186.
"VALUE" OF CONCENTRATION NUMBER  2 =

1204.
"VALUE" OF CONCENTRATION NUMBER  3 =

826.
"VALUE" OF CONCENTRATION NUMBER  3 =

628.
"VALUE" OF CONCENTRATION NUMBER  3 =

816.
"VALUE" OF CONCENTRATION NUMBER  4 =

493.
"VALUE" OF CONCENTRATION NUMBER  4 =

416.
"VALUE" OF CONCENTRATION NUMBER  4 =

413.
"VALUE" OF CONCENTRATION NUMBER  5 =

127.
"VALUE" OF CONCENTRATION NUMBER  5 =

147.
"VALUE" OF CONCENTRATION NUMBER  5 =
  147.
"VALUE" OF CONCENTRATION NUMBER  6 =

44.
"VALUE" OF CONCENTRATION NUMBER  6 =

40.
"VALUE" OF CONCENTRATION NUMBER  6 =

49.3
```

2.3.3 Computer Generated Listings of Input and Output from Computer Program for Dunnett's Procedure (Continued)

```
  R E S U L T S   O F   S E Q U E N T I A L   C O M P A R I S O N S
          U S I N G   T H E   D U N N E T T S   T E S T
------------------------------------------------------------------

 FOR THIS SET OF DATA, THE MINIMUM DIFFERENCE THAT CAN BE
 DETECTED AS STATISTICALLY SIGNIFICANT IS      225.339
 THIS REPRESENTS A  18.1% REDUCTION IN THE MEAN
 RESPONSE FROM THE CONTROL.
 T =  2.50

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 2 AND CONTROL.

=> THERE IS SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 3 AND CONTROL.

=> THERE IS SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 4 AND CONTROL.

=> THERE IS SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 5 AND CONTROL.

=> THERE IS SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION 6 AND CONTROL.

                      I N P U T   D A T A   T A B L E
--------------------------------------------------------------

  1209.000   1212.000    826.000    493.000    107.000     44.000
  1180.000   1186.000    628.000    416.000    147.000     40.000
  1340.000   1204.000    816.000    413.000    147.000     49.300

             T R A N S F O R M E D   D A T A   T A B L E
--------------------------------------------------------------
NOTE: TRANSFORMATION NUMBER 3 WAS USED FOR THIS SET OF DATA

     3.082      3.084      2.917      2.693      2.104      1.643
     3.072      3.074      2.798      2.619      2.167      1.602
     3.127      3.081      2.912      2.616      2.167      1.693
--------------------------------------------------------------


                    A N O V A   T A B L E
                    ---------------------

 SOURCE     DF          SUM OF SQ.      MEAN SQ.        CALC F   TAB F(0.05)
------------------------------------------------------------------
AMONG        5             4.995          0.999        560.183     3.110
WITHIN      12             0.021          0.002
------------------------------------------------------------------
TOTAL       17             5.016
------------------------------------------------------------------
```

2.4 Listing of Computer Program for Dunnett's Procedure

```
PDS> TY DUNNETT.FTN
       REAL VALUE(30,22),A12(22),A(22),A1(22),A8,A9,M(22),M1,
      + X,Y(22),Y1,C,TTLSS,TRSS,ERRSS,AMMS,WIMS,D,T,CL1,CL2,FRATIO,
      + D1,D51,VAL(30,22),A6(30,22),A61(30,22),A5(30,22),A51(30,22)
      +,S56,XBAR(22),ORG,M12(22),AZ,AZ1,FR1
C
       DIMENSION T(7,28),FR(7,43)
C
       DATA T/2.02,2.44,2.68,2.85,2.98,3.08,3.16,1.94,2.34,2.56,2.71,
      *2.83,2.92,3.,1.89,2.27,2.48,2.62,2.73,2.82,2.89,1.86,2.22,2.42,2.5
      *5,2.66,2.74,2.81,1.83,2.18,2.37,2.5,2.6,2.68,2.75,1.81,2.15,2.34,
      *2.47,2.56,2.64,2.7,1.8,2.13,2.31,2.44,2.53,2.6,2.67,1.78,2.11,2.2
      *9,2.41,2.5,2.58,2.64,1.77,2.09,2.27,2.39,2.48,2.55,2.61,1.76,2.08
      *,2.25,2.37,2.46,2.53,2.59,1.75,2.07,2.24,2.36,2.44,2.51,2.57,1.75
      *,2.06,2.23,2.34,2.43,2.5,2.56,1.74,2.05,2.22,2.33,2.42,2.49,2.54
      *,1.73,2.04,2.21,2.32,2.41,2.48,2.53,1.73,2.03,2.2,2.31,2.4,2.47,
      *2.52,1.72,2.03,2.19,2.3,2.39,2.46,2.51,1.72,2.03,2.19,2.3,2.39,2.
      *46,2.51,1.72,2.03,2.19,2.3,2.38,2.45,2.5,1.71,2.02,2.18,2.29,2.38
      *,2.45,2.5,1.71,2.01,2.17,2.28,2.36,2.43,2.48,1.71,2.01,2.17,2.27,
      *2.36,2.43,2.48,1.71,2.01,2.17,2.27,2.35,2.42,2.47,1.71,2.,2.16,2
      *.27,2.35,2.41,2.46,1.71,2.,2.16,2.26,2.34,2.41,2.45,1.7,2.,2.16,
      *2.26,2.34,2.41,2.45,1.7,1.99,2.15,2.25,2.33,2.4,2.45,1.7,1.99,2.
      *15,2.25,2.33,2.4,2.45,1.7,1.99,2.15,2.25,2.33,2.4,2.45/
C
       DATA FR/6.61,5.79,5.41,5.19,5.05,4.95,4.88,5.99,5.14,4.76,4.53,
      *4.39,4.28,4.21,5.59,4.74,4.35,4.12,3.97,3.87,3.79,5.32,4.46,4.07,
      *3.84,3.69,3.58,3.5,5.12,4.26,3.86,3.63,3.48,3.37,3.29,4.96,4.1,3.7
      *1,3.48,3.33,3.22,3.14,4.84,3.98,3.59,3.36,3.2,3.09,3.01,4.75,3.88,
      *3.49,3.26,3.11,3.,2.92,4.67,3.8,3.41,3.18,3.02,2.92,2.84,4.6,3.74,
      *3.34,3.11,2.96,2.85,2.77,4.54,3.68,3.29,3.06,2.9,2.79,2.7,4.49,3.6
      *3,3.24,3.01,2.85,2.74,2.66,4.45,3.59,3.2,2.96,2.81,2.7,2.62,4.41,
      *3.55,3.16,2.93,2.77,2.66,2.58,4.38,3.52,3.13,2.9,2.74,2.63,2.55,
      *4.35,3.49,3.1,2.87,2.71,2.6,2.52,4.32,3.47,3.07,2.84,2.68,2.57,
      *2.49,4.3,3.44,3.05,2.82,2.66,2.55,2.47,4.28,3.42,3.03,2.8,2.64,2.5
      *3,2.45,4.26,3.4,3.01,2.78,2.62,2.51,2.43,4.24,3.38,2.99,2.76,2.6,
      *2.49,2.41,4.22,3.37,2.98,2.74,2.59,2.47,2.39,4.21,3.35,2.96,2.73,
      *2.57,2.46,2.37,4.2,3.34,2.95,2.71,2.56,2.44,2.36,4.18,3.33,2.93,
      *2.7,2.54,2.43,2.35,4.17,3.32,2.92,2.69,2.53,2.42,2.34,4.16,3.31,2.
      *91,2.68,2.52,2.41,2.33,4.15,3.3,2.9,2.67,2.51,2.4,2.32,4.14,3.29,
      *2.89,2.66,2.5,2.39,2.31,4.13,3.28,2.88,2.65,2.49,2.38,2.3,4.11,
      *3.26,2.86,2.63,2.48,2.36,2.28,4.1,3.25,2.85,2.62,2.47,2.35,2.27,
      *4.1,3.25,2.85,2.62,2.46,2.35,2.26,4.09,3.24,2.84,2.61,2.45,2.34,
      *2.25,4.08,3.23,2.84,2.61,2.45,2.34,2.25,4.08,3.22,2.83,2.6,2.44,
      *2.33,2.24,4.07,3.22,2.83,2.59,2.44,2.32,2.24,4.06,3.21,2.83,2.59,
      *2.43,2.32,2.23,4.06,3.21,2.82,2.58,2.43,2.31,2.23,4.05,3.2,2.81,
      *2.58,2.42,2.3,2.23,4.05,3.2,2.81,2.57,2.42,2.3,2.22,4.04,3.2,2.8
      *,2.56,2.41,2.3,2.22,4.04,3.19,2.8,2.56,2.41,2.3,2.21/
C
       INTEGER R1,R9,R(10),CONC1,CS,TAIL,ASI,EN,OK,R5,
      +NAME(36),D69,VALU
C
       TYPE*,' '
       TYPE*,' '
       TYPE 653
   653 FORMAT(' IF YOU NEED INSTRUCTIONS,TYPE 1. OTHERWISE,TYPE 0 - ',$)
       ACCEPT 655,INSTR
   655 FORMAT(A3)
       IF(INSTR.EQ.'0')GO TO 991
       TYPE*,' '
       TYPE*,'ANOVA-WITH DUNNETTS COMPARISON OF EACH CONCENTRATION MEAN'
       TYPE*,'VS THE CONTROL MEAN. UP TO EIGHT CONCENTRATIONS (INCLUDING'
       TYPE*,'CONTROL) MAY BE USED.'
       TYPE*,' '
       TYPE*,' A ONE-SIDED "T" AT 95% IS USED IN ALL CASES.'
       TYPE*,' '
       TYPE*,' '
       TYPE*,' THE FOLLOWING TRANSFORMATIONS ARE AVAILABLE TO USE ON'
       TYPE*,' THE VALUES:'
       TYPE*,' '
       TYPE*,' NUMBER      TRANSFORMATION'
       TYPE*,'   0         NONE'
       TYPE*,'   1         SQUARE ROOT(X)'
       TYPE*,'   3         LOG 10(X)'
```

2.4 Listing of Program for Dunnett's Procedure (Continued)

```
      TYPE*,'   5       (ARCSIN(SQ. RT(X)/(N+1)+'
      TYPE*,'            ARCSIN(SQ. RT(X+1)/(N+1)))/2'
      TYPE*,'   6        ARCSIN((X)**0.5)'
      TYPE*,'            N O T E  :'
      TYPE*,'                      THE LIMITS OF SIN ARE -1. AND +1.'
  991 TYPE 72
   72 FORMAT(' TRANSFORMATION NUMBER = ',$)
      ACCEPT 73,NUMBER
   73 FORMAT(A3)
      IF(NUMBER.EQ.'6')TYPE 750
      IF(NUMBER.EQ.'6')ACCEPT 752,ORG
      TYPE 5
    5 FORMAT(' INCLUDING CONTROL, NUMBER OF CONCENTRATIONS = ',$)
      ACCEPT 7,CONC1
    7 FORMAT(I2)
      TYPE*,'NOTE: BEGIN WITH CONTROL(CONCENTRATION NUMBER 1) AND ENTER
     1 IN ASCENDING ORDER.'
      TYPE*,' '
      TYPE*,' '
      DO 11,I=1,CONC1
      WRITE(5,8),I
    8 FORMAT(' NUMBER OF REPLICATES IN CONCENTRATION NUMBER  ',I2)
      TYPE 9
    9 FORMAT(' = ',$)
      ACCEPT 10,R(I)
   10 FORMAT(I3)
      R1=R1+R(I)
   11 CONTINUE
      R15=R1/CONC1
      TYPE*,' '
      TYPE*,' '
      DO 3 I=1,CONC1
      JJ=1
      R9=R(1)
      DO 4 J=2,CONC1
      IF(R(J)-R9)4,4,6
    6 R9=R(J)
      JJ=J
    4 CONTINUE
    3 CONTINUE
   12 FORMAT(I3)
      TYPE*,' '
  802 TYPE*,' NOTE: DECIMAL POINT MUST BE USED IN "VALUE".'
      TYPE*,' '
      TYPE*,' '
      DO 30,I=1,CONC1
      DO 25,J=1,R(I)
  659 WRITE(5,13),I
   13 FORMAT(' "VALUE" OF CONCENTRATION NUMBER ',I2,' = ')
      TYPE 15
   15 FORMAT( $)
      ACCEPT 17,VALUE(I,J)
   17 FORMAT(F17.4)
    2 IF(NUMBER.EQ.'3')GO TO 751
      IF(NUMBER.EQ.'6')GO TO 18
      IF(NUMBER.EQ.'1')GO TO 630
      IF(NUMBER.EQ.'5')GO TO 899
      IF(NUMBER.EQ.'0')GC TO 753
      IF(NUMBER.EQ.'7')GO TO 753
  751 VAL(I,J)=ALOG10(VALUE(I,J))
      A(I)=A(I)+VAL(I,J)
      A12(I)=A12(I)+VALUE(I,J)
      A1(I)=A1(I)+(VAL(I,J)**2)
      GO TO 25
  899 TYPE 750
  750 FORMAT(' AVERAGE NUMBER OF TEST ORGANISMS AT
     1 BEGINNING PER CONCENTRATION = ',$)
      ACCEPT 752,ORG
  752 FORMAT(F2.0)
      A6(I,J)=(VALUE(I,J)/(ORG+1))**0.5
      A12(I)=A12(I)+VALUE(I,J)
      A61(I,J)=ASIN(A6(I,J))*180./3.1415927
      A5(I,J)=((VALUE(I,J)+1)/(ORG+1))**0.5
      A51(I,J)=ASIN(A5(I,J))*180./3.1415927
      VAL(I,J)=0.5*(A51(I,J)+A61(I,J))
      A(I)=A(I)+VAL(I,J)
      A12(I)=A12(I)+VALUE(I,J)
      A1(I)=A1(I)+(VAL(I,J)**2)
      GO TO 25
  630 VAL(I,J)=VALUE(I,J)**0.5
      A(I)=A(I)+VAL(I,J)
      A12(I)=A12(I)+VALUE(I,J)
      A1(I)=A1(I)+(VAL(I,J)**2)
      GO TO 25
   18 V=ASIN(1/(4*ORG))**0.5
      V1=ASIN(((ORG-1./4.)/ORG)**0.5)
      VAL(I,J)=ASIN(VALUE(I,J)**0.5)
      IF(VALUE(I,J).EQ.0.)VAL(I,J)=V
      IF(VALUE(I,J).EQ.1.)VAL(I,J)=V1
      A(I)=A(I)+VAL(I,J)
      TYPE*,' TRANSFORMED VALUE (ARCSIN TRANSFORMATION) ='
      WRITE(5,17)VAL(I,J)
      A12(I)=A12(I)+VALUE(I,J)
      A1(I)=A1(I)+(VAL(I,J)**2)
      GO TO 25
  753 VAL(I,J)=VALUE(I,J)
      A(I)=A(I)+VAL(I,J)
      A1(I)=A1(I)+(VAL(I,J)**2)
      A12(I)=A12(I)+VALUE(I,J)
   25 CONTINUE
   30 CONTINUE
      DO 50,I=1,CONC1
      A8=A8+A(I)
      A9=A9+A1(I)
   60 CONTINUE
      M1=A8/R1
      DO 70,I=1,CONC1
      XBAR(I)=A(I)**2/R(I)
      X=X+XBAR(I)
   70 CONTINUE
      DO 80,I=1,CONC1
      Y(I)=A1(I)-XBAR(I)
   80 CONTINUE
      DO 85,I=1,CONC1
      Y1=Y1+Y(I)
   85 CONTINUE
      C=A8**2/R1
      TTLSS=A9-C
       DO 90,I=1,CONC1
      TRSS=A(I)**2/R(I)+TRSS
   90 CONTINUE
      TRSS=TRSS-C
      ERRSS=TTLSS-TRSS
      AMMS=TRSS/(CONC1-1)
      WIMS=ERRSS/(R1-CONC1)
      S56=((A9-X)/(R1-CONC1))**0.5
      FRATIO=AMMS/WIMS
      R5=R1-CONC1
      C5=CONC1-1
      D69=R1-CONC1
      FR1=FR(C5,D69-4)
      IF(D69.GT.32)GO TO 555
      IF(C5.GT.7)GO TO 555
      TT=T(C5,D69-4)
      GO TO 556
  555 TYPE*,'THE "T-TABLE" AND "F-TABLE" ARE PARTS OF THIS PROGRAM.HOW-'
      TYPE*,' EVER THEY ARE PROGRAMMED ONLY UP TO A LEVEL OF EIGHT'
      TYPE*,' CONCENTRATIONS (INCLUDING CONTROL) AND TO NO MORE THAN '
      TYPE*,' SIX REPLICATES. SINCE THIS SET OF DATA EXCEEDS THESE '
      TYPE*,' LIMITS, PLEASE LOOK UP A TABLE OF T FOR ONE-SIDED '
      TYPE*,' COMPARISONS. IT IS AVAILABLE IN C. W. DUNNETTS - '
      TYPE*,' "A MULTIPLE COMPARISON PROCEDURE FOR COMPARING SEVERAL '
      TYPE*,' TREATMENTS WITH A CONTROL". JOURNAL OF THE AMERICAN '
      TYPE*,' STATISTICAL ASSOCIATION, 1955, 50, 1096-1121. ALSO, LOOK '
      TYPE*,' UP AN F - TABLE (FOUND IN MOST STATISTICS BOOKS). '
      WRITE(5,557)C5,D69
  557 FORMAT(' FOR ',I3,' CONCENTRATIONS AND',I3,' DEGREES OF FREEDOM
```

2.4 Listing of Program for Dunnett's Procedure (Continued)

```
     1 (AT 95%)')
      TYPE 558
  558 FORMAT( ' T = ',$)
      ACCEPT 559,TT
  559 FORMAT(F4.2)
      WRITE(5,557)CS,D69
      TYPE 567
  567 FORMAT(  ' F = ',$)
      ACCEPT 559,FR1
      TYPE*,' '
  556 WRITE(5,915)
      TYPE*,'             U S I N G   T H E   D U N N E T T S   T E S T'
  850 FORMAT( '  T = ',F5.2)
      TYPE*,'________________________________________________________________
     1__________'
      TYPE*,' '
  200 FORMAT('  DETECTED AS STATISTICALLY SIGNIFICANT IS ',F11.3,' .')
  201 FORMAT('  THIS REPRESENTS A',F6.1,'% REDUCTION IN THE MEAN')
      A2=R(1)
      IF(NUMBER.EQ.'1')D51=D1**2
      IF(NUMBER.EQ.'3')D51=10.**D1
      IF(NUMBER.EQ.'7')D51=D1
      IF(NUMBER.EQ.'6')D51=SIN(D1)**2
      IF(NUMBER.EQ.'5')D51=SIN(D1)**2
      TYPE*,' '
      D55=M(1)-D1
      D56=(SIN(D55)**2)
      D57=(SIN(M(1))**2)
      D58=D57-D56
      D59=D58/M12(1)*100.
      D60=10.**D55
      D61=M12(1)-D60
      D62=D61/M12(1)*100.
      IF(NUMBER.EQ.'3')D58=D61
      IF(NUMBER.EQ.'3')D59=D62
      TYPE*,' FOR THIS SET OF DATA, THE MINIMUM DIFFERENCE THAT CAN BE'
      WRITE(5,200)D58
      WRITE(5,201)D59
      TYPE*,' RESPONSE FROM THE CONTROL.'
      WRITE(5,850)TT
      TYPE*,' '
  914 FORMAT(I3)
  915 FORMAT('   R E S U L T S   O F   S E Q U E N T I A L   C O M P A
     1R I S O N S')
      DO 401,I=2,CONC1
      IF(ABS(M(I)-M(1)).GT.D1)GO TO 350
      WRITE(5,912)I
  912 FORMAT(' THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONCENTRAT
     1ION', I2,' AND CONTROL.')
      GO TO 401
  350 TYPE*,' '
      WRITE(5,370),I
  370 FORMAT(' => THERE IS SIGNIFICANT DIFFERENCE BETWEEN CONCENTRATION'
     1,I2,' AND CONTROL.' )
  401 CONTINUE
      TYPE*,' '
      TYPE*,' '
      TYPE*,'                          I N P U T   D A T A   T A B L E'
      TYPE*,'________________________________________________________________
     1________'
      TYPE*,' '
      DO 995,I=1,R9
      WRITE(5,98),(VALUE(J,I),J=1,CONC1)
   98 FORMAT(12F11.3)
  995 CONTINUE
      TYPE*,' '
      TYPE*,' '
      IF(NUMBER.EQ.'0')GO TO 700
      IF(NUMBER.EQ.'7')GO TO 700
```

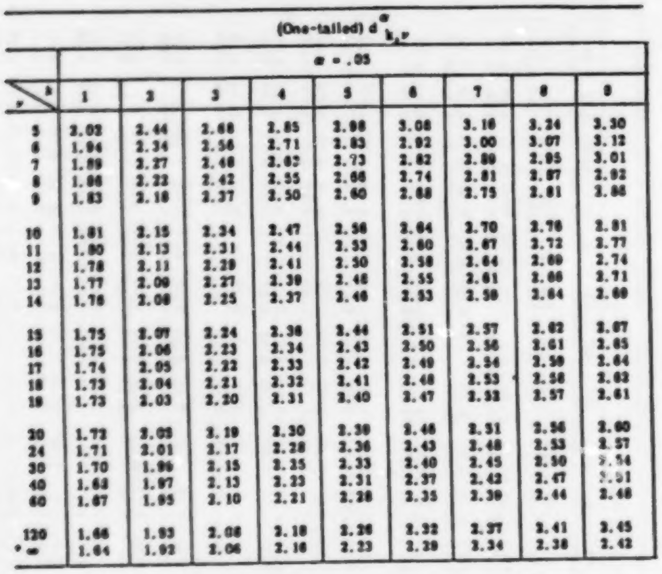
2.4 Listing of Program for Dunnett's Procedure (Continued)

```
      TYPE*,'              T R A N S F O R M E D   D A T A   T A B L E'
      TYPE*,'----------------------------------------------------------
     1------'
      WRITE(5,702)NUMBER
  702 FORMAT(' NOTE: TRANSFORMATION NUMBER',A3,'WAS USED FOR THIS
     1 SET OF DATA')
      TYPE*,' '
      DO 701,I=1,R9
      WRITE(5,98),(VAL(J,I),J=1,CONC1)
  701 CONTINUE
  700 TYPE*,'------------------------------------------------------------
     1------'
      TYPE*,'                     --------------------'
      TYPE*,' '
      TYPE*,' '
      WRITE(5,96)
      TYPE*,'--------------------------------------------------------
     1--------------'
   96 FORMAT('  SOURCE',5X,'DF',8X,'SUM OF SQ.',3X,'   MEAN SQ.'
     1,3X,'     CALC F   TAB F(0.05)')
      WRITE(5,91),CONC1-1,TRSS,AMMS,FRATIO,FR1
      WRITE(5,92),R1-CONC1,ERRSS,WIMS
      TYPE*,'--------------------------------------------------------
     1--------------'
      WRITE(5,93),R1-1,TTLSS
   91 FORMAT(' AMONG',5X,I3,2X,2(F15.3),6X,2(F9.3,1X))
   92 FORMAT(' WITHIN',4X,I3,3X,2(F14.3,1X))
   93 FORMAT(' TOTAL',5X,I3,1X,F16.3)
      TYPE*,'------------------------------------------------------------
     1---------'
  954 TYPE*,' '
      TYPE*,' '
      WRITE(5,841)
      WRITE(5,841)
  841 FORMAT(////////////////)
  654 STOP
      END

PDS>
```

2.5 Table of Dunnett's "t" values.

TABLE C.5. DUNNETT'S "T" VALUES[1]



(One-tailed) $d^{\alpha}_{k,\nu}$

	α = .05								
ν \ k	1	2	3	4	5	6	7	8	9
5	2.02	2.44	2.68	2.85	2.98	3.08	3.16	3.24	3.30
6	1.94	2.34	2.56	2.71	2.83	2.92	3.00	3.07	3.12
7	1.89	2.27	2.48	2.62	2.73	2.82	2.89	2.95	3.01
8	1.86	2.22	2.42	2.55	2.66	2.74	2.81	2.87	2.92
9	1.83	2.18	2.37	2.50	2.60	2.68	2.75	2.81	2.86
10	1.81	2.15	2.34	2.47	2.56	2.64	2.70	2.76	2.81
11	1.80	2.13	2.31	2.44	2.53	2.60	2.67	2.72	2.77
12	1.78	2.11	2.29	2.41	2.50	2.58	2.64	2.69	2.74
13	1.77	2.09	2.27	2.39	2.48	2.55	2.61	2.66	2.71
14	1.76	2.08	2.25	2.37	2.46	2.53	2.59	2.64	2.69
15	1.75	2.07	2.24	2.36	2.44	2.51	2.57	2.62	2.67
16	1.75	2.06	2.23	2.34	2.43	2.50	2.56	2.61	2.65
17	1.74	2.05	2.22	2.33	2.42	2.49	2.54	2.59	2.64
18	1.73	2.04	2.21	2.32	2.41	2.48	2.53	2.58	2.62
19	1.73	2.03	2.20	2.31	2.40	2.47	2.52	2.57	2.61
20	1.72	2.03	2.19	2.30	2.39	2.46	2.51	2.56	2.60
24	1.71	2.01	2.17	2.28	2.36	2.43	2.48	2.53	2.57
30	1.70	1.99	2.15	2.25	2.33	2.40	2.45	2.50	2.54
40	1.68	1.97	2.13	2.23	2.31	2.37	2.42	2.47	2.51
60	1.67	1.95	2.10	2.21	2.28	2.35	2.39	2.44	2.48
120	1.66	1.93	2.08	2.18	2.26	2.32	2.37	2.41	2.45
*∞	1.64	1.92	2.06	2.16	2.23	2.29	2.34	2.38	2.42

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[1] From: Miller, 1981.

2.6 Table of "F" values.

TABLE C.6. FIVE PERCENT (UPPER) AND ONE PERCENT (LOWER) VALUES FOR THE DISTRIBUTION OF "F".[1]

n_2	n_1 degrees of freedom (for greater mean square)																								n_2
	1	2	3	4	5	6	7	8	9	10	11	12	14	16	20	24	30	40	50	75	100	200	500	∞	
1	161 4,052	200 4,999	216 5,403	225 5,625	230 5,764	234 5,859	237 5,928	239 5,981	241 6,022	242 6,056	243 6,082	244 6,106	245 6,142	246 6,169	248 6,208	249 6,234	250 6,258	251 6,286	252 6,302	253 6,323	253 6,334	254 6,352	254 6,361	254 6,366	1
2	18.51 98.49	19.00 99.00	19.16 99.17	19.25 99.25	19.30 99.30	19.33 99.33	19.36 99.34	19.37 99.36	19.38 99.38	19.39 99.40	19.40 99.41	19.41 99.42	19.42 99.43	19.43 99.44	19.44 99.45	19.45 99.46	19.46 99.47	19.47 99.48	19.47 99.48	19.48 99.49	19.49 99.49	19.49 99.49	19.50 99.50	19.50 99.50	2
3	10.13 34.12	9.55 30.82	9.28 29.46	9.12 28.71	9.01 28.24	8.94 27.91	8.88 27.67	8.84 27.49	8.81 27.34	8.78 27.23	8.76 27.13	8.74 27.05	8.71 26.92	8.69 26.83	8.66 26.69	8.64 26.60	8.62 26.50	8.60 26.41	8.58 26.35	8.57 26.27	8.56 26.23	8.54 26.18	8.54 26.14	8.53 26.12	3
4	7.71 21.20	6.94 18.00	6.59 16.69	6.39 15.98	6.26 15.52	6.16 15.21	6.09 14.98	6.04 14.80	6.00 14.66	5.96 14.54	5.93 14.45	5.91 14.37	5.87 14.24	5.84 14.15	5.80 14.02	5.77 13.93	5.74 13.83	5.71 13.74	5.70 13.69	5.68 13.61	5.66 13.57	5.65 13.52	5.64 13.48	5.63 13.46	4
5	6.61 16.26	5.79 13.27	5.41 12.06	5.19 11.39	5.05 10.97	4.95 10.67	4.88 10.45	4.82 10.27	4.78 10.15	4.74 10.05	4.70 9.96	4.68 9.89	4.64 9.77	4.60 9.68	4.56 9.55	4.53 9.47	4.50 9.38	4.46 9.29	4.44 9.24	4.42 9.17	4.40 9.13	4.38 9.07	4.37 9.04	4.36 9.02	5
6	5.99 13.74	5.14 10.92	4.76 9.78	4.53 9.15	4.39 8.75	4.28 8.47	4.21 8.26	4.15 8.10	4.10 7.98	4.06 7.87	4.03 7.79	4.00 7.72	3.96 7.60	3.92 7.52	3.87 7.39	3.84 7.31	3.81 7.23	3.77 7.14	3.75 7.09	3.72 7.02	3.71 6.99	3.69 6.94	3.68 6.90	3.67 6.88	6
7	5.59 12.25	4.74 9.55	4.35 8.45	4.12 7.85	3.97 7.46	3.87 7.19	3.79 7.00	3.73 6.84	3.68 6.71	3.63 6.62	3.60 6.54	3.57 6.47	3.52 6.35	3.49 6.27	3.44 6.15	3.41 6.07	3.38 5.98	3.34 5.90	3.32 5.85	3.29 5.78	3.28 5.75	3.25 5.70	3.24 5.67	3.23 5.65	7
8	5.32 11.26	4.46 8.65	4.07 7.59	3.84 7.01	3.69 6.63	3.58 6.37	3.50 6.19	3.44 6.03	3.39 5.91	3.34 5.82	3.31 5.74	3.28 5.67	3.23 5.56	3.20 5.48	3.15 5.36	3.12 5.28	3.08 5.20	3.05 5.11	3.03 5.06	3.00 5.00	2.98 4.96	2.96 4.91	2.94 4.88	2.93 4.86	8
9	5.12 10.56	4.26 8.02	3.86 6.99	3.63 6.42	3.48 6.06	3.37 5.80	3.29 5.62	3.23 5.47	3.18 5.35	3.13 5.26	3.10 5.18	3.07 5.11	3.02 5.00	2.98 4.92	2.93 4.80	2.90 4.73	2.86 4.64	2.82 4.56	2.80 4.51	2.77 4.45	2.76 4.41	2.73 4.36	2.72 4.33	2.71 4.31	9
10	4.96 10.04	4.10 7.56	3.71 6.55	3.48 5.99	3.33 5.64	3.22 5.39	3.14 5.21	3.07 5.06	3.02 4.95	2.97 4.85	2.94 4.78	2.91 4.71	2.86 4.60	2.82 4.52	2.77 4.41	2.74 4.33	2.70 4.25	2.67 4.17	2.64 4.12	2.61 4.05	2.59 4.01	2.56 3.96	2.55 3.93	2.54 3.91	10
11	4.84 9.65	3.98 7.20	3.59 6.22	3.36 5.67	3.20 5.32	3.09 5.07	3.01 4.88	2.95 4.74	2.90 4.63	2.86 4.54	2.82 4.46	2.79 4.40	2.74 4.29	2.70 4.21	2.65 4.10	2.61 4.02	2.57 3.94	2.53 3.86	2.50 3.80	2.47 3.74	2.45 3.70	2.42 3.66	2.41 3.62	2.40 3.60	11
12	4.75 9.33	3.88 6.93	3.49 5.95	3.26 5.41	3.11 5.06	3.00 4.82	2.92 4.65	2.85 4.50	2.80 4.39	2.76 4.30	2.72 4.22	2.69 4.16	2.64 4.05	2.60 3.98	2.54 3.86	2.50 3.78	2.46 3.70	2.42 3.61	2.40 3.56	2.36 3.49	2.35 3.46	2.32 3.41	2.31 3.38	2.30 3.36	12
13	4.67 9.07	3.80 6.70	3.41 5.74	3.18 5.20	3.02 4.86	2.92 4.62	2.84 4.44	2.77 4.30	2.72 4.19	2.67 4.10	2.63 4.02	2.60 3.96	2.55 3.85	2.51 3.78	2.46 3.67	2.42 3.59	2.38 3.51	2.34 3.42	2.32 3.37	2.28 3.30	2.26 3.27	2.24 3.21	2.22 3.18	2.21 3.16	13

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[1] From Snedecor and Cochran, 1980.

TABLE C.6. FIVE PERCENT (UPPER) AND ONE PERCENT (LOWER) VALUES FOR THE DISTRIBUTION OF "F" (CONTINUED).

f_2	f_1 degrees of freedom (for greater mean square)																								f_2
	1	2	3	4	5	6	7	8	9	10	11	12	14	16	20	24	30	40	50	75	100	200	500	∞	
14	4.60 8.86	3.74 6.51	3.34 5.56	3.11 5.03	2.96 4.69	2.85 4.46	2.77 4.28	2.70 4.14	2.65 4.03	2.60 3.94	2.56 3.86	2.53 3.80	2.48 3.70	2.44 3.62	2.39 3.51	2.35 3.43	2.31 3.34	2.27 3.26	2.24 3.21	2.21 3.14	2.19 3.11	2.16 3.06	2.14 3.02	2.13 3.00	14
15	4.54 8.68	3.68 6.36	3.29 5.42	3.06 4.89	2.90 4.56	2.79 4.32	2.70 4.14	2.64 4.00	2.59 3.89	2.55 3.80	2.51 3.73	2.48 3.67	2.43 3.56	2.39 3.48	2.33 3.36	2.29 3.29	2.25 3.20	2.21 3.12	2.18 3.07	2.15 3.00	2.12 2.97	2.10 2.92	2.08 2.89	2.07 2.87	15
16	4.49 8.53	3.63 6.23	3.24 5.29	3.01 4.77	2.85 4.44	2.74 4.20	2.66 4.03	2.59 3.89	2.54 3.78	2.49 3.69	2.45 3.61	2.42 3.55	2.37 3.45	2.33 3.37	2.28 3.25	2.24 3.18	2.20 3.10	2.16 3.01	2.13 2.96	2.09 2.89	2.07 2.86	2.04 2.80	2.02 2.77	2.01 2.75	16
17	4.45 8.40	3.59 6.11	3.20 5.18	2.96 4.67	2.81 4.34	2.70 4.10	2.62 3.93	2.55 3.79	2.50 3.68	2.45 3.59	2.41 3.52	2.38 3.45	2.33 3.35	2.29 3.27	2.23 3.16	2.19 3.08	2.15 3.00	2.11 2.92	2.08 2.86	2.04 2.79	2.02 2.76	1.99 2.70	1.97 2.67	1.96 2.65	17
18	4.41 8.28	3.55 6.01	3.16 5.09	2.93 4.58	2.77 4.25	2.66 4.01	2.58 3.85	2.51 3.71	2.46 3.60	2.41 3.51	2.37 3.44	2.34 3.37	2.29 3.27	2.25 3.19	2.19 3.07	2.15 3.00	2.11 2.91	2.07 2.83	2.04 2.78	2.00 2.71	1.98 2.68	1.95 2.62	1.93 2.59	1.92 2.57	18
19	4.38 8.18	3.52 5.93	3.13 5.01	2.90 4.50	2.74 4.17	2.63 3.94	2.55 3.77	2.48 3.63	2.43 3.52	2.38 3.43	2.34 3.36	2.31 3.30	2.26 3.19	2.21 3.12	2.15 3.00	2.11 2.92	2.07 2.84	2.02 2.76	2.00 2.70	1.96 2.63	1.94 2.60	1.91 2.54	1.90 2.51	1.88 2.49	19
20	4.35 8.10	3.49 5.85	3.10 4.94	2.87 4.43	2.71 4.10	2.60 3.87	2.52 3.71	2.45 3.56	2.40 3.45	2.35 3.37	2.31 3.30	2.28 3.23	2.23 3.13	2.18 3.05	2.12 2.94	2.08 2.86	2.04 2.77	1.99 2.69	1.96 2.63	1.92 2.56	1.90 2.53	1.87 2.47	1.85 2.44	1.84 2.42	20
21	4.32 8.02	3.47 5.78	3.07 4.87	2.84 4.37	2.68 4.04	2.57 3.81	2.49 3.65	2.42 3.51	2.37 3.40	2.32 3.31	2.28 3.24	2.25 3.17	2.20 3.07	2.15 2.99	2.09 2.88	2.05 2.80	2.00 2.72	1.96 2.63	1.93 2.58	1.89 2.51	1.87 2.47	1.84 2.42	1.82 2.38	1.81 2.36	21
22	4.30 7.94	3.44 5.72	3.05 4.82	2.82 4.31	2.66 3.99	2.55 3.76	2.47 3.59	2.40 3.45	2.35 3.35	2.30 3.26	2.26 3.18	2.23 3.12	2.18 3.02	2.13 2.94	2.07 2.83	2.03 2.75	1.98 2.67	1.93 2.58	1.91 2.53	1.87 2.46	1.84 2.42	1.81 2.37	1.80 2.33	1.78 2.31	22
23	4.28 7.88	3.42 5.66	3.03 4.76	2.80 4.26	2.64 3.94	2.53 3.71	2.45 3.54	2.38 3.41	2.32 3.30	2.28 3.21	2.24 3.14	2.20 3.07	2.14 2.97	2.10 2.89	2.04 2.78	2.00 2.70	1.96 2.62	1.91 2.53	1.88 2.48	1.84 2.41	1.82 2.37	1.79 2.32	1.77 2.28	1.76 2.26	23
24	4.26 7.82	3.40 5.61	3.01 4.72	2.78 4.22	2.62 3.90	2.51 3.67	2.43 3.50	2.36 3.36	2.30 3.25	2.26 3.17	2.22 3.09	2.18 3.03	2.13 2.93	2.09 2.85	2.02 2.74	1.98 2.66	1.94 2.58	1.89 2.49	1.86 2.44	1.82 2.36	1.80 2.33	1.76 2.27	1.74 2.23	1.73 2.21	24
25	4.24 7.77	3.38 5.57	2.99 4.68	2.76 4.18	2.60 3.86	2.49 3.63	2.41 3.46	2.34 3.32	2.28 3.21	2.24 3.13	2.20 3.05	2.16 2.99	2.11 2.89	2.06 2.81	2.00 2.70	1.96 2.62	1.92 2.54	1.87 2.45	1.84 2.40	1.80 2.32	1.77 2.29	1.74 2.23	1.72 2.19	1.71 2.17	25
26	4.22 7.72	3.37 5.53	2.98 4.64	2.74 4.14	2.59 3.82	2.47 3.59	2.39 3.42	2.32 3.29	2.27 3.17	2.22 3.09	2.18 3.02	2.15 2.96	2.10 2.86	2.05 2.77	1.99 2.66	1.95 2.58	1.90 2.50	1.85 2.41	1.82 2.36	1.78 2.28	1.76 2.25	1.72 2.19	1.70 2.15	1.69 2.13	26

TABLE C.6. FIVE PERCENT (UPPER) AND ONE PERCENT (LOWER)
VALUES FOR THE DISTRIBUTION OF "F" (CONTINUED).

f_2	f_1 degrees of freedom (for greater mean square)																								f_2
	1	2	3	4	5	6	7	8	9	10	11	12	14	16	20	24	30	40	50	75	100	200	500	∞	
27	4.21 7.68	3.35 5.49	2.96 4.60	2.73 4.11	2.57 3.79	2.46 3.56	2.37 3.39	2.30 3.26	2.25 3.14	2.20 3.06	2.16 2.98	2.13 2.93	2.08 2.83	2.03 2.74	1.97 2.63	1.93 2.55	1.88 2.47	1.84 2.38	1.80 2.33	1.76 2.25	1.74 2.21	1.71 2.16	1.68 2.12	1.67 2.10	27
28	4.20 7.64	3.34 5.45	2.95 4.57	2.71 4.07	2.56 3.76	2.44 3.53	2.36 3.36	2.29 3.23	2.24 3.11	2.19 3.03	2.15 2.95	2.12 2.90	2.06 2.80	2.02 2.71	1.96 2.60	1.91 2.52	1.87 2.44	1.81 2.35	1.78 2.30	1.75 2.22	1.72 2.18	1.69 2.13	1.67 2.09	1.65 2.06	28
29	4.18 7.60	3.33 5.42	2.93 4.54	2.70 4.04	2.54 3.73	2.43 3.50	2.35 3.33	2.28 3.20	2.22 3.08	2.18 3.00	2.14 2.92	2.10 2.87	2.05 2.77	2.00 2.68	1.94 2.57	1.90 2.49	1.85 2.41	1.80 2.32	1.77 2.27	1.73 2.19	1.71 2.15	1.68 2.10	1.65 2.06	1.64 2.03	29
30	4.17 7.56	3.32 5.39	2.92 4.51	2.69 4.02	2.53 3.70	2.42 3.47	2.34 3.30	2.27 3.17	2.21 3.06	2.16 2.98	2.12 2.90	2.09 2.84	2.04 2.74	1.99 2.66	1.93 2.55	1.89 2.47	1.84 2.38	1.79 2.29	1.76 2.24	1.72 2.16	1.69 2.13	1.66 2.07	1.64 2.03	1.62 2.01	30
32	4.15 7.50	3.30 5.34	2.90 4.46	2.67 3.97	2.51 3.66	2.40 3.42	2.32 3.25	2.25 3.12	2.19 3.01	2.14 2.94	2.10 2.86	2.07 2.80	2.02 2.70	1.97 2.62	1.91 2.51	1.86 2.42	1.82 2.34	1.76 2.25	1.74 2.20	1.69 2.12	1.67 2.08	1.64 2.02	1.61 1.98	1.59 1.96	32
34	4.13 7.44	3.28 5.29	2.88 4.42	2.65 3.93	2.49 3.61	2.38 3.38	2.30 3.21	2.23 3.08	2.17 2.97	2.12 2.89	2.08 2.82	2.05 2.76	2.00 2.66	1.95 2.58	1.89 2.47	1.84 2.38	1.80 2.30	1.74 2.21	1.71 2.15	1.67 2.08	1.64 2.04	1.61 1.98	1.59 1.94	1.57 1.91	34
36	4.11 7.39	3.26 5.25	2.86 4.38	2.63 3.89	2.48 3.58	2.36 3.35	2.28 3.18	2.21 3.04	2.15 2.94	2.10 2.86	2.06 2.78	2.03 2.72	1.98 2.62	1.93 2.54	1.87 2.43	1.82 2.35	1.78 2.26	1.72 2.17	1.69 2.12	1.65 2.04	1.62 2.00	1.59 1.94	1.56 1.90	1.55 1.87	36
38	4.10 7.35	3.25 5.21	2.85 4.34	2.62 3.86	2.46 3.54	2.35 3.32	2.26 3.15	2.19 3.02	2.14 2.91	2.09 2.82	2.05 2.75	2.02 2.69	1.96 2.59	1.92 2.51	1.85 2.40	1.80 2.32	1.76 2.22	1.71 2.14	1.67 2.08	1.63 2.00	1.60 1.97	1.57 1.90	1.54 1.86	1.53 1.84	38
40	4.08 7.31	3.23 5.18	2.84 4.31	2.61 3.83	2.45 3.51	2.34 3.29	2.25 3.12	2.18 2.99	2.12 2.88	2.07 2.80	2.04 2.73	2.00 2.66	1.95 2.56	1.90 2.49	1.84 2.37	1.79 2.29	1.74 2.20	1.69 2.11	1.66 2.05	1.61 1.97	1.59 1.94	1.55 1.88	1.53 1.84	1.51 1.81	40
42	4.07 7.27	3.22 5.15	2.83 4.29	2.59 3.80	2.44 3.49	2.32 3.26	2.24 3.10	2.17 2.96	2.11 2.86	2.06 2.77	2.02 2.70	1.99 2.64	1.94 2.54	1.89 2.46	1.82 2.35	1.78 2.26	1.73 2.17	1.68 2.08	1.64 2.02	1.60 1.94	1.57 1.91	1.54 1.85	1.51 1.80	1.49 1.78	42
44	4.06 7.24	3.21 5.12	2.82 4.26	2.58 3.78	2.43 3.46	2.31 3.24	2.23 3.07	2.16 2.94	2.10 2.84	2.05 2.75	2.01 2.68	1.98 2.62	1.92 2.52	1.88 2.44	1.81 2.32	1.76 2.24	1.72 2.15	1.66 2.06	1.63 2.00	1.58 1.92	1.56 1.88	1.52 1.82	1.50 1.78	1.48 1.75	44
46	4.05 7.21	3.20 5.10	2.81 4.24	2.57 3.76	2.42 3.44	2.30 3.22	2.22 3.05	2.14 2.92	2.09 2.82	2.04 2.73	2.00 2.66	1.97 2.60	1.91 2.50	1.87 2.42	1.80 2.30	1.75 2.22	1.71 2.13	1.65 2.04	1.62 1.98	1.57 1.90	1.54 1.86	1.51 1.80	1.48 1.76	1.46 1.72	46
48	4.04 7.19	3.19 5.08	2.80 4.22	2.56 3.74	2.41 3.42	2.30 3.20	2.21 3.04	2.14 2.90	2.08 2.80	2.03 2.71	1.99 2.64	1.96 2.58	1.90 2.48	1.86 2.40	1.79 2.28	1.74 2.20	1.70 2.11	1.64 2.02	1.61 1.96	1.56 1.88	1.53 1.84	1.50 1.78	1.47 1.73	1.45 1.70	48

TABLE C.6. FIVE PERCENT (UPPER) AND ONE PERCENT (LOWER) VALUES FOR THE DISTRIBUTION OF "F" (CONTINUED).

	*f*₁ degrees of freedom (for greater mean square)																								
*f*₂	1	2	3	4	5	6	7	8	9	10	11	12	14	16	20	24	30	40	50	75	100	200	500	∞	*f*₂
50	4.03 7.17	3.18 5.06	2.79 4.20	2.56 3.72	2.40 3.41	2.29 3.18	2.20 3.02	2.13 2.88	2.07 2.78	2.02 2.70	1.98 2.62	1.95 2.56	1.90 2.46	1.85 2.39	1.78 2.26	1.74 2.18	1.69 2.10	1.63 2.00	1.60 1.94	1.55 1.86	1.52 1.82	1.48 1.76	1.46 1.71	1.44 1.68	50
55	4.02 7.12	3.17 5.01	2.78 4.16	2.54 3.68	2.38 3.37	2.27 3.15	2.18 2.98	2.11 2.85	2.05 2.75	2.00 2.66	1.97 2.59	1.93 2.53	1.88 2.43	1.83 2.35	1.76 2.23	1.72 2.15	1.67 2.06	1.61 1.96	1.58 1.90	1.52 1.82	1.50 1.78	1.46 1.71	1.43 1.66	1.41 1.64	55
60	4.00 7.08	3.15 4.98	2.76 4.13	2.52 3.65	2.37 3.34	2.25 3.12	2.17 2.95	2.10 2.82	2.04 2.72	1.99 2.63	1.95 2.56	1.92 2.50	1.86 2.40	1.81 2.32	1.75 2.20	1.70 2.12	1.65 2.03	1.59 1.93	1.56 1.87	1.50 1.79	1.48 1.74	1.44 1.68	1.41 1.63	1.39 1.60	60
65	3.99 7.04	3.14 4.95	2.75 4.10	2.51 3.62	2.36 3.31	2.24 3.09	2.15 2.93	2.08 2.79	2.02 2.70	1.98 2.61	1.94 2.54	1.90 2.47	1.85 2.37	1.80 2.30	1.73 2.18	1.68 2.09	1.63 2.00	1.57 1.90	1.54 1.84	1.49 1.76	1.46 1.71	1.42 1.64	1.39 1.60	1.37 1.56	65
70	3.98 7.01	3.13 4.92	2.74 4.08	2.50 3.60	2.35 3.29	2.23 3.07	2.14 2.91	2.07 2.77	2.01 2.67	1.97 2.59	1.93 2.51	1.89 2.45	1.84 2.35	1.79 2.28	1.72 2.15	1.67 2.07	1.62 1.98	1.56 1.88	1.53 1.82	1.47 1.74	1.45 1.69	1.40 1.62	1.37 1.56	1.35 1.53	70
80	3.96 6.96	3.11 4.88	2.72 4.04	2.48 3.56	2.33 3.25	2.21 3.04	2.12 2.87	2.05 2.74	1.99 2.64	1.95 2.55	1.91 2.48	1.88 2.41	1.82 2.32	1.77 2.24	1.70 2.11	1.65 2.03	1.60 1.94	1.54 1.84	1.51 1.78	1.45 1.70	1.42 1.65	1.38 1.57	1.35 1.52	1.32 1.49	80
100	3.94 6.90	3.09 4.82	2.70 3.98	2.46 3.51	2.30 3.20	2.19 2.99	2.10 2.82	2.03 2.69	1.97 2.59	1.92 2.51	1.88 2.43	1.85 2.36	1.79 2.26	1.75 2.19	1.68 2.06	1.63 1.98	1.57 1.89	1.51 1.79	1.48 1.73	1.42 1.64	1.39 1.59	1.34 1.51	1.30 1.46	1.28 1.43	100
125	3.92 6.84	3.07 4.78	2.68 3.94	2.44 3.47	2.29 3.17	2.17 2.95	2.08 2.79	2.01 2.65	1.95 2.56	1.90 2.47	1.86 2.40	1.83 2.33	1.77 2.23	1.72 2.15	1.65 2.03	1.60 1.94	1.55 1.85	1.49 1.75	1.45 1.68	1.39 1.59	1.36 1.54	1.31 1.46	1.27 1.40	1.25 1.37	125
150	3.91 6.81	3.06 4.75	2.67 3.91	2.43 3.44	2.27 3.14	2.16 2.92	2.07 2.76	2.00 2.62	1.94 2.53	1.89 2.44	1.85 2.37	1.82 2.30	1.76 2.20	1.71 2.12	1.64 2.00	1.59 1.91	1.54 1.83	1.47 1.72	1.44 1.66	1.37 1.56	1.34 1.51	1.29 1.43	1.25 1.37	1.22 1.33	150
200	3.89 6.76	3.04 4.71	2.65 3.88	2.41 3.41	2.26 3.11	2.14 2.90	2.05 2.73	1.98 2.60	1.92 2.50	1.87 2.41	1.83 2.34	1.80 2.28	1.74 2.17	1.69 2.09	1.62 1.97	1.57 1.88	1.52 1.79	1.45 1.69	1.42 1.62	1.35 1.53	1.32 1.48	1.26 1.39	1.22 1.33	1.19 1.28	200
400	3.86 6.70	3.02 4.66	2.62 3.83	2.39 3.36	2.23 3.06	2.12 2.85	2.03 2.69	1.96 2.55	1.90 2.46	1.85 2.37	1.81 2.29	1.78 2.23	1.72 2.12	1.67 2.04	1.60 1.92	1.54 1.84	1.49 1.74	1.42 1.64	1.38 1.57	1.32 1.47	1.28 1.42	1.22 1.32	1.16 1.24	1.13 1.19	400
1000	3.85 6.66	3.00 4.62	2.61 3.80	2.38 3.34	2.22 3.04	2.10 2.82	2.02 2.66	1.95 2.53	1.89 2.43	1.84 2.34	1.80 2.26	1.76 2.20	1.70 2.09	1.65 2.01	1.58 1.89	1.53 1.81	1.47 1.71	1.41 1.61	1.36 1.54	1.30 1.44	1.26 1.38	1.19 1.28	1.13 1.19	1.08 1.11	1000
∞	3.84 6.64	2.99 4.60	2.60 3.78	2.37 3.32	2.21 3.02	2.09 2.80	2.01 2.64	1.94 2.51	1.88 2.41	1.83 2.32	1.79 2.24	1.75 2.18	1.69 2.07	1.64 1.99	1.57 1.87	1.52 1.79	1.46 1.69	1.40 1.59	1.35 1.52	1.28 1.41	1.24 1.36	1.17 1.25	1.11 1.15	1.00 1.00	∞

APPENDIX D

STEEL'S MANY-ONE RANK TEST[1]

1. Steel's Many-One Rank Test is a nonparametric test for comparing treatments with a control. This test is an alternative to the Dunnett's test, and may be applied to the data when the normality assumption has not been met. Steel's test requires equal variances across the treatments and the control, but it is thought to be fairly insensitive to deviations from this condition (Steel, 1959). The tables for Steel's Test require an equal number of replicates at each concentration. If this is not the case, critical values can be approximated by interpolation.

2. An example of the use of this test is provided below. The test employs reproduction data taken from Table 2, Section 13, Ceriodaphnia survival and reproduction test. The reproduction data for 25% effluent were omitted from the analysis because the results of Fisher's Exact Test indicated that a significant proportion of the organisms died at that concentration. Since mortality is a significant effect, the remaining task was to determine if there were significant differences in reproduction at the lower effluent concentrations.

3. For each control and concentration conbination, combine the data and arrange the observations in order of size from smallest to largest. Assign the ranks (1,2,3, . . . 20) to the ordered observations (1 to the smallest, 2 to the next smallest, etc.). If ties occur in the ranking, assign the average rank to the observation.

4. An example of assigning ranks to the combined data for the control and concentration 1% effluent is given in Table D.1 below. This ranking procedure is repeated for each control and concentration combination. The complete set of rankings is listed in Table D.2. The ranks are then summed for each effluent concentration, as shown in Table D.3.

5. For this set of data, we wish to determine if the reproduction in any of the effluent concentrations is significantly lower than the reproduction by the control organisms. If this occurs, the rank sum at that concentration would be significantly lower than the rank sum of the control. Thus, we are only concerned with comparing the rank sums for the reproduction of each of the various effluent concentrations with some "minimum" or critical rank sum, at or below which the reproduction would be considered to be significantly lower than the control. At a probability level of 0.05, the minimum rank sum in a test with four concentrations and 10 replicates per concentration, is 76 (see Table D.4).

6. None of the rank sums in Table D.3 is less than or equal to 76. Therefore, none of the four effluent concentrations reduced reproduction significantly below that of the controls, and the NOEC is assumed to be 12% effluent.

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TABLE D.1. EXAMPLE OF STEEL'S MANY-ONE RANK TEST: ASSIGNING RANKS TO THE CONTROL AND 1% EFFLUENT CONCENTRATION

Rank	Number of Young Produced	Control or % Effluent
1	0	Control
2	6	1%
3	8	Control
4	11	1%
5	15	1%
6.5	16	Control
6.5	16	Control
8	18	Control
9	21	1%
11	22	1%
11	22	1%
11	22	Control
14	23	1%
14	23	1%
14	23	1%
16	24	Control
17	25	Control
18	26	Control
19	27	Control
20	31	1%

TABLE D.2. TABLE OF RANKS

Replicate (Organism)	Control	Effluent Concentration			
		1%	3%	6%	12%
A	22 (11,11.5,13.5,13)	22 (11)	25 (17)	23 (15)	20 (11)
B	18 (8,6.5,8,9,)	15 (5)	25 (17)	16 (6)	21 (12)
C	27 (19,20,20,20)	21 (9)	20 (8.5)	7 (2)	17 (7)
D	16 (6.5,4.5,6,5)	6 (2)	24 (14.5)	13 (4)	13 (3)
E	16 (6.5,4.5,6,5)	23 (14)	15 (3)	26 (18.5)	25 (16.5)
F	0 (1,1,1,1)	31 (20)	23 (13)	22 (13.5)	25 (16.5)
G	8 (3,2,3,2)	23 (14)	21 (10)	21 (10.5)	16 (5)
H	25 (17,17,17,16.5)	11 (4)	22 (11.5)	21 (10.5)	25 (16.5)
I	24 (16,14.5,16,14)	23 (14)	20 (8.5)	21 (10.5)	18 (9)
J	26 (18,19,18.5,19)	22 (11)	18 (6.5)	21 (10.5)	18 (9)

Control ranks are given in the order of the concentration with which they were ranked.

TABLE D.3. RANK SUMS

Effluent Concentration (%)	Rank Sum
1	104.0
3	109.5
6	101.0
12	105.5

TABLE D.4. SIGNIFICANT VALUES OF RANK SUMS: JOINT CONFIDENCE COEFFICIENTS OF 0.95 (UPPER) and 0.99 (LOWER) FOR ONE-SIDED ALTERNATIVES

	k = number of treatments (excluding control)							
n	2	3	4	5	6	7	8	9
4	11	10	10	10	10	—	—	—
	—	—	—	—	—	—	—	—
5	18	17	17	16	16	16	16	15
	15	—	—	—	—	—	—	—
6	27	26	25	25	24	24	24	23
	23	22	21	21	—	—	—	—
7	37	36	35	35	34	34	33	33
	32	31	30	30	29	29	29	29
8	49	48	47	46	46	45	45	44
	43	42	41	40	40	40	39	39
9	63	62	61	60	59	59	58	58
	56	55	54	53	52	52	51	51
10	79	77	76	75	74	74	73	72
	71	69	68	67	66	66	65	65
11	97	95	93	92	91	90	90	89
	87	85	84	83	82	81	81	80
12	116	114	112	111	110	109	108	108
	105	103	102	100	99	99	98	98
13	138	135	133	132	130	129	129	128
	125	123	121	120	119	118	117	117
14	161	158	155	154	153	152	151	150
	147	144	142	141	140	139	138	137
15	186	182	180	178	177	176	175	174
	170	167	165	164	162	161	160	160
16	213	209	206	204	203	201	200	199
	196	192	190	188	187	186	185	184
17	241	237	234	232	231	229	228	227
	223	219	217	215	213	212	211	210
18	272	267	264	262	260	259	257	256
	252	248	245	243	241	240	239	238
19	304	299	296	294	292	290	288	287
	282	278	275	273	271	270	268	267
20	339	333	330	327	325	323	322	320
	315	310	307	305	303	301	300	299

From Steel, 1959.

APPENDIX E

FISHER'S EXACT TEST[1]

1. Fisher's Exact Test (Finney, 1948; Pearson and Hartley, 1962) is a statistical method based on the hypergeometric probability distribution that can be used to test if the proportion of successes is the same in two Bernoulli (binomial) populations. When used with the *Ceriodaphnia* data, it provides a conservative test of the equality of any two survival proportions assuming only the independence of responses from a Bernoulli population.

2. The data for this example (Table E.1) are mortality data from the *Ceriodaphnia* survival and reproduction test, discussed in Section 13 of this manual.

3. For each control and effluent concentration construct a 2x2 contingency table as follows:

	Number of		Number of Observations
	Successes	Failures	
Condition 1	a	A - a	A
Condition 2	b	B - b	B
Total	a + b	[(A+B) - a - b]	A + B

4. Arrange the table so that $A \geq B$. Categorize a success such that $a/A \geq b/B$. For this data, a success may be 'alive' or 'dead' whichever causes $a/A \geq b/B$. The test is then conducted by looking up a value in the table of significance levels of b and comparing it to the b value given in the contingency table. The table of significance levels of b is included in this example, Table E.4. Enter Table E.4 in the section for A, subsection for B, and the line for a. If the b value of the contingency table is equal to or less than the integer in the column headed 0.05, a/A is significantly greater than b/B. A dash or absence of entry in Table E.4 indicates that no contingency table in that class is significant.

5. For the control and effluent concentration of 1% the appropriate contingency table for the test is given in Table E.2.

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TABLE E.1. EXAMPLE OF FISHER'S EXACT TEST: CERIODAPHNIA MORTALITY DATA

Effluent Concentration (%)	No. Dead	Total*
Control	1	9
1	0	10
3	0	10
6	0	10
12	0	10
25	10	10

*Total number of live adults at the beginning of the test.

TABLE E.2. 2X2 CONTINGENCY TABLE FOR CONTROL AND 1% EFFLUENT

	Number of		Number of Observations
	Alive	Dead	
1% Effluent	10	0	10
Control	8	1	9
Total	18	1	19

6. Since 10/10 $\geq$ 8/9, the category 'alive' is regarded as a success. For A = 10, B = 9 and, a = 10, under the column headed 0.05, the value from Table E.4 is b = 5. Since the value of b (b = 8) from the contingency table (Table E.2), is greater than the value of b (b = 5) from Table E.4, the test concludes that the proportion of survival is not significantly different for the control and 1% effluent.

7. The contingency tables for the combinations of control and effluent concentrations of 3%, 6%, 12% are identical to Table E.2. The conclusion of no significant difference in the proportion of survival for the control and the level of effluent would also remain the same.

8. For the combination of control and 25% effluent, the contingency table would be constructed as Table E.3. The category 'dead' is regarded as a success, since 10/10 > 1/9. The b value (b = 1) from the contingency table (Table E.3) is less than the b value (b = 5) from the table of significance levels of b (Table E.4). Thus, the percent mortality for 25% effluent is significantly greater than the percent mortality for the control.

Table E.3. 2X2 CONTINGENCY TABLE FOR CONTROL AND 25% EFFLUENT

	Number of		Number of Observations
	Dead	Alive	
25% Effluent	10	0	10
Control	1	8	9
Total	11	8	19

TABLE E.4. SIGNIFICANT LEVELS OF B AND CORRESPONDING PROBABILITIES[1]

	a	Probability			
		0·05	0·025	0·01	0·005
A=3 B=3	3	**0** ·050	—	—	—
A=4 B=4	4	**0** ·014	**0** ·014	—	—
3	4	**0** ·029	—	—	—
A=5 B=5	5	**1** ·024	**1** ·024	**0** ·004	**0** ·004
	4	**0** ·024	**0** ·024	—	—
4	5	**1** ·048	**0** ·008	**0** ·008	—
	4	**0** ·040	—	—	—
3	5	**0** ·018	**0** ·018	—	—
2	5	**0** ·048	—	—	—
A=6 B=6	6	**2** ·030	**1** ·008	**1** ·008	**0** ·001
	5	**1** ·040	**0** ·008	**0** ·008	—
	4	**0** ·030	—	—	—
5	6	**1** ·015+	**0** ·015+	**0** ·002	**0** ·002
	5	**0** ·013	**0** ·013	—	—
	4	**0** ·045+	—	—	—
4	6	**1** ·033	**0** ·005−	**0** ·005−	**0** ·005−
	5	**0** ·024	**0** ·024	—	—
3	6	**0** ·012	**0** ·012	—	—
	5	**0** ·048	—	—	—
2	6	**0** ·036	—	—	—
A=7 B=7	7	**3** ·035−	**2** ·010+	**1** ·002	**1** ·002
	6	**1** ·015−	**1** ·015−	**0** ·002	**0** ·002
	5	**0** ·010+	**0** ·010+	—	—
	4	**0** ·035−	—	—	—
6	7	**2** ·021	**2** ·021	**1** ·005−	**1** ·005−
	6	**1** ·025+	**0** ·004	**0** ·004	**0** ·004
	5	**0** ·016	**0** ·016	—	—
	4	**0** ·049	—	—	—
5	7	**2** ·045+	**1** ·010+	**0** ·001	**0** ·001
	6	**1** ·045+	**0** ·008	**0** ·008	—
	5	**0** ·027	—	—	—
4	7	**1** ·024	**1** ·024	**0** ·003	**0** ·003
	6	**0** ·015+	**0** ·015+	—	—
	5	**0** ·045+	—	—	—
3	7	**0** ·008	**0** ·008	**0** ·008	—
	6	**0** ·033	—	—	—
2	7	**0** ·028	—	—	—
A=8 B=8	8	**4** ·038	**3** ·013	**2** ·003	**2** ·003
	7	**2** ·020	**2** ·020	**1** ·005+	**0** ·001
	6	**1** ·020	**1** ·020	**0** ·003	**0** ·003
	5	**0** ·013	**0** ·013	—	—
	4	**0** ·038	—	—	—
7	8	**3** ·026	**2** ·007	**2** ·007	**1** ·001
	7	**2** ·035−	**1** ·009	**1** ·009	**0** ·001
	6	**1** ·032	**0** ·006	**0** ·006	—
	5	**0** ·019	**0** ·019	—	—
6	8	**2** ·015−	**2** ·015−	**1** ·003	**1** ·003
	7	**1** ·016	**1** ·016	**0** ·002	**0** ·002
	6	**0** ·009	**0** ·009	**0** ·009	—
	5	**0** ·028	—	—	—
5	8	**2** ·035−	**1** ·007	**1** ·007	**0** ·001
	7	**1** ·032	**0** ·005−	**0** ·005−	**0** ·005−
	6	**0** ·016	**0** ·016	—	—
	5	**0** ·044	—	—	—
4	8	**1** ·018	**1** ·018	**0** ·002	**0** ·002
	7	**0** ·010+	**0** ·010+	—	—
	6	**0** ·030	—	—	—
3	8	**0** ·006	**0** ·006	**0** ·006	—
	7	**0** ·024	**0** ·024	—	—
2	8	**0** ·022	**0** ·022	—	—
A=9 B=9	9	**5** ·041	**4** ·015−	**3** ·005−	**3** ·005−
	8	**3** ·025−	**3** ·025−	**2** ·008	**1** ·002
	7	**2** ·028	**1** ·008	**1** ·008	**0** ·001
	6	**1** ·025−	**1** ·025−	**0** ·005−	**0** ·005−
	5	**0** ·015−	**0** ·015−	—	—
	4	**0** ·041	—	—	—
8	9	**4** ·029	**3** ·009	**3** ·009	**2** ·002
	8	**3** ·043	**2** ·013	**1** ·003	**1** ·003
	7	**2** ·044	**1** ·012	**0** ·002	**0** ·002
	6	**1** ·036	**0** ·007	**0** ·007	—
	5	**0** ·020	**0** ·020	—	—
7	9	**3** ·019	**3** ·019	**2** ·005−	**2** ·005−
	8	**2** ·024	**2** ·024	**1** ·006	**0** ·001
	7	**1** ·020	**1** ·020	**0** ·003	**0** ·003
	6	**0** ·010+	**0** ·010+	—	—
	5	**0** ·029	—	—	—
6	9	**3** ·044	**2** ·011	**1** ·002	**1** ·002
	8	**2** ·047	**1** ·011	**0** ·001	**0** ·001
	7	**1** ·035−	**0** ·006	**0** ·006	—
	6	**0** ·017	**0** ·017	—	—
	5	**0** ·042	—	—	—

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[1] The table shows:(1) In bold type, for given a, A and B, the value of b (<a) which is just significant at the probability level quoted (one-tailed test); and (2) In small type, for given A, B and r = a + b, the exact probability (if there is independence) that b is equal to or less than the integer shown in bold type. From Pearson and Hartley, 1962.

TABLE E.4. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE
AND CORRESPONDING PROBABILITIES (SMALL TYPE) (CONTINUED).

	a	Probability			
		0·05	0·025	0·01	0·005
A=9 B=5	9	2 ·027	1 ·005−	1 ·005−	1 ·005−
	8	1 ·023	1 ·023	0 ·003	0 ·003
	7	0 ·010+	0 ·010+	—	—
	6	0 ·028	—	—	—
4	9	1 ·014	1 ·014	0 ·001	0 ·001
	8	0 ·007	0 ·007	0 ·007	—
	7	0 ·021	0 ·021	—	—
	6	0 ·049	—	—	—
3	9	1 ·045+	0 ·005−	0 ·005−	0 ·005−
	8	0 ·018	0 ·018	—	—
	7	0 ·045+	—	—	—
2	9	0 ·018	0 ·018	—	—
A=10 B=10	10	6 ·043	5 ·016	4 ·005+	3 ·002
	9	4 ·029	3 ·010−	3 ·010−	2 ·003
	8	3 ·035−	2 ·012	1 ·003	1 ·003
	7	2 ·035−	1 ·010−	1 ·010−	0 ·002
	6	1 ·029	0 ·005+	0 ·005+	—
	5	0 ·016	0 ·016	—	—
	4	0 ·043	—	—	—
9	10	5 ·033	4 ·011	3 ·003	3 ·003
	9	4 ·050−	3 ·017	2 ·005−	2 ·005−
	8	2 ·019	2 ·019	1 ·004	1 ·004
	7	1 ·015−	1 ·015−	0 ·002	0 ·002
	6	1 ·040	0 ·008	0 ·008	—
	5	0 ·022	0 ·022	—	—
8	10	4 ·023	4 ·023	3 ·007	2 ·002
	9	3 ·032	2 ·009	2 ·009	1 ·002
	8	2 ·031	1 ·008	1 ·008	0 ·001
	7	1 ·023	1 ·023	0 ·004	0 ·004
	6	0 ·011	0 ·011	—	—
	5	0 ·029	—	—	—
7	10	3 ·015−	3 ·015−	2 ·003	2 ·003
	9	2 ·018	2 ·018	1 ·004	1 ·004
	8	1 ·013	1 ·013	0 ·002	0 ·002
	7	1 ·036	0 ·006	0 ·006	—
	6	0 ·017	0 ·017	—	—
	5	0 ·041	—	—	—
6	10	3 ·036	2 ·008	2 ·008	1 ·001
	9	2 ·036	1 ·008	1 ·008	0 ·001
	8	1 ·024	1 ·024	0 ·003	0 ·003
	7	0 ·010+	0 ·010+	—	—
	6	0 ·026	—	—	—
5	10	2 ·022	2 ·022	1 ·004	1 ·004
	9	1 ·017	1 ·017	0 ·002	0 ·002
	8	1 ·047	0 ·007	0 ·007	—
	7	0 ·019	0 ·019	—	—
	6	0 ·042	—	—	—
A=10 B=4	10	1 ·011	1 ·011	0 ·001	0 ·001
	9	1 ·041	0 ·005−	0 ·005−	0 ·005−
	8	0 ·015−	0 ·015−	—	—
	7	0 ·035−	—	—	—
3	10	1 ·038	0 ·003	0 ·003	0 ·003
	9	0 ·014	0 ·014	—	—
	8	0 ·035−	—	—	—
2	10	0 ·015+	0 ·015+	—	—
	9	0 ·045+	—	—	—
A=11 B=11	11	7 ·045+	6 ·018	5 ·006	4 ·002
	10	5 ·032	4 ·012	3 ·004	3 ·004
	9	4 ·040	3 ·015−	2 ·004	2 ·004
	8	3 ·043	2 ·015−	1 ·004	1 ·004
	7	2 ·040	1 ·012	0 ·002	0 ·002
	6	1 ·032	0 ·006	0 ·006	—
	5	0 ·018	0 ·018	—	—
	4	0 ·045+	—	—	—
10	11	6 ·035+	5 ·012	4 ·004	4 ·004
	10	4 ·021	4 ·021	3 ·007	2 ·002
	9	3 ·024	3 ·024	2 ·007	1 ·002
	8	2 ·023	2 ·023	1 ·006	0 ·001
	7	1 ·017	1 ·017	0 ·003	0 ·003
	6	1 ·043	0 ·009	0 ·009	—
	5	0 ·023	0 ·023	—	—
9	11	5 ·026	4 ·008	4 ·008	3 ·002
	10	4 ·038	3 ·012	2 ·003	2 ·003
	9	3 ·040	2 ·012	1 ·003	1 ·003
	8	2 ·035−	1 ·009	1 ·009	0 ·001
	7	1 ·025−	1 ·025−	0 ·004	0 ·004
	6	0 ·012	0 ·012	—	—
	5	0 ·030	—	—	—
8	11	4 ·018	4 ·018	3 ·005−	3 ·005−
	10	3 ·024	3 ·024	2 ·006	1 ·001
	9	2 ·022	2 ·022	1 ·005−	1 ·005−
	8	1 ·015−	1 ·015−	0 ·002	0 ·002
	7	1 ·037	0 ·007	0 ·007	—
	6	0 ·017	0 ·017	—	—
	5	0 ·040	—	—	—
7	11	4 ·043	3 ·011	2 ·002	2 ·002
	10	3 ·047	2 ·013	1 ·002	1 ·002
	9	2 ·039	1 ·009	1 ·009	0 ·001
	8	1 ·025−	1 ·025−	0 ·004	0 ·004
	7	0 ·010+	0 ·010+	—	—
	6	0 ·025−	0 ·025−	—	—
6	11	3 ·029	2 ·006	2 ·006	1 ·001
	10	2 ·028	1 ·005+	1 ·005+	0 ·001
	9	1 ·018	1 ·018	0 ·002	0 ·002

TABLE E.4. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE AND CORRESPONDING PROBABILITIES (SMALL TYPE) (CONTINUED).

	a	Probability			
		0·05	0·025	0·01	0·005
A=11 B=6	8	1 ·043	0 ·007	0 ·007	—
	7	0 ·017	0 ·017	—	—
	6	0 ·037	—	—	—
5	11	2 ·018	2 ·018	1 ·003	1 ·003
	10	1 ·013	1 ·013	0 ·001	0 ·001
	9	1 ·036	0 ·005⁻	0 ·005⁻	0 ·005⁻
	8	0 ·013	0 ·013	—	—
	7	0 ·029	—	—	—
4	11	1 ·009	1 ·009	1 ·009	0 ·001
	10	1 ·033	0 ·004	0 ·004	0 ·004
	9	0 ·011	0 ·011	—	—
	8	0 ·026	—	—	—
3	11	1 ·033	0 ·003	0 ·003	0 ·003
	10	0 ·011	0 ·011	—	—
	9	0 ·027	—	—	—
2	11	0 ·013	0 ·013	—	—
	10	0 ·038	—	—	—
A=12 B=12	12	8 ·047	7 ·019	6 ·007	5 ·002
	11	6 ·034	5 ·014	4 ·005⁻	4 ·005⁻
	10	5 ·045⁻	4 ·018	3 ·006	2 ·002
	9	4 ·050⁻	3 ·020	2 ·006	1 ·001
	8	3 ·050⁻	2 ·018	1 ·005⁻	1 ·005⁻
	7	2 ·045⁻	1 ·014	0 ·002	0 ·002
	6	1 ·034	0 ·007	0 ·007	—
	5	0 ·019	0 ·019	—	—
	4	0 ·047	—	—	—
11	12	7 ·037	6 ·014	5 ·005⁻	5 ·005⁻
	11	5 ·024	5 ·024	4 ·008	3 ·002
	10	4 ·029	3 ·010⁺	2 ·003	2 ·003
	9	3 ·030	2 ·009	2 ·009	1 ·002
	8	2 ·026	1 ·007	1 ·007	0 ·001
	7	1 ·019	1 ·019	0 ·003	0 ·003
	6	1 ·045⁻	0 ·009	0 ·009	—
	5	0 ·024	0 ·024	—	—
10	12	6 ·029	5 ·010⁻	5 ·010⁻	4 ·003
	11	5 ·043	4 ·015⁺	3 ·005⁻	3 ·005⁻
	10	4 ·048	3 ·017	2 ·005⁻	2 ·005⁻
	9	3 ·046	2 ·015⁻	1 ·004	1 ·004
	8	2 ·038	1 ·010⁺	0 ·002	0 ·002
	7	1 ·026	0 ·005⁻	0 ·005⁻	0 ·005⁻
	6	0 ·012	0 ·012	—	—
	5	0 ·030	—	—	—
9	12	5 ·021	5 ·021	4 ·006	3 ·002
	11	4 ·029	3 ·009	3 ·009	2 ·002
	10	3 ·029	2 ·008	2 ·008	1 ·002
	9	2 ·024	2 ·024	1 ·006	0 ·001
	8	1 ·016	1 ·016	0 ·002	0 ·002
A=12 B=9	7	1 ·037	0 ·007	0 ·007	—
	6	0 ·017	0 ·017	—	—
	5	0 ·039	—	—	—
8	12	5 ·049	4 ·014	3 ·004	3 ·004
	11	3 ·018	3 ·018	2 ·004	2 ·004
	10	2 ·015⁺	2 ·015⁺	1 ·003	1 ·003
	9	2 ·040	1 ·010⁻	1 ·010⁻	0 ·001
	8	1 ·025⁻	1 ·025⁻	0 ·004	0 ·004
	7	0 ·010⁻	0 ·010⁻	—	—
	6	0 ·024	0 ·024	—	—
7	12	4 ·036	3 ·009	3 ·009	2 ·002
	11	3 ·038	2 ·010⁻	2 ·010⁻	1 ·002
	10	2 ·029	1 ·006	1 ·006	0 ·001
	9	1 ·017	1 ·017	0 ·002	0 ·002
	8	1 ·040	0 ·007	0 ·007	—
	7	0 ·016	0 ·016	—	—
	6	0 ·034	—	—	—
6	12	3 ·025⁻	3 ·025⁻	2 ·005⁻	2 ·005⁻
	11	2 ·022	2 ·022	1 ·004	1 ·004
	10	1 ·013	1 ·013	0 ·002	0 ·002
	9	1 ·032	0 ·005⁻	0 ·005⁻	0 ·005⁻
	8	0 ·011	0 ·011	—	—
	7	0 ·025⁻	0 ·025⁻	—	—
	6	0 ·050⁻	—	—	—
5	12	2 ·015⁻	2 ·015⁻	1 ·002	1 ·002
	11	1 ·010⁻	1 ·010⁻	1 ·010⁻	0 ·001
	10	1 ·028	0 ·003	0 ·003	0 ·003
	9	0 ·009	0 ·009	0 ·009	—
	8	0 ·020	0 ·020	—	—
	7	0 ·041	—	—	—
4	12	2 ·050	1 ·007	1 ·007	0 ·001
	11	1 ·027	0 ·003	0 ·003	0 ·003
	10	0 ·008	0 ·008	0 ·008	—
	9	0 ·019	0 ·019	—	—
	8	0 ·038	—	—	—
3	12	1 ·029	0 ·002	0 ·002	0 ·002
	11	0 ·009	0 ·009	0 ·009	—
	10	0 ·022	0 ·022	—	—
	9	0 ·044	—	—	—
2	12	0 ·011	0 ·011	—	—
	11	0 ·033	—	—	—
A=13 B=13	13	9 ·048	8 ·020	7 ·007	6 ·003
	12	7 ·037	6 ·015⁺	5 ·006	4 ·002
	11	6 ·048	5 ·031	4 ·008	3 ·002
	10	4 ·024	4 ·024	3 ·008	2 ·002
	9	3 ·024	3 ·024	2 ·008	1 ·002
	8	2 ·021	2 ·021	1 ·006	0 ·001

TABLE E.4. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE AND CORRESPONDING PROBABILITIES (SMALL TYPE) (CONTINUED).

	a	Probability			
		0·05	0·025	0·01	0·005
A=13 B=13	7	2 ·048	1 ·015+	0 ·003	0 ·003
	6	1 ·037	0 ·007	0 ·007	—
	5	0 ·020	0 ·020	—	—
	4	0 ·048	—	—	—
12	13	8 ·039	7 ·015−	6 ·005+	5 ·002
	12	6 ·027	5 ·010−	5 ·010−	4 ·003
	11	5 ·033	4 ·013	3 ·004	3 ·004
	10	4 ·036	3 ·013	2 ·004	2 ·004
	9	3 ·034	2 ·011	1 ·003	1 ·003
	8	2 ·029	1 ·008	1 ·008	0 ·001
	7	1 ·020	1 ·020	0 ·004	0 ·004
	6	1 ·046	0 ·010−	0 ·010−	—
	5	0 ·024	0 ·024	—	—
11	13	7 ·031	6 ·011	5 ·003	5 ·003
	12	6 ·048	5 ·018	4 ·006	3 ·002
	11	4 ·021	4 ·021	3 ·007	2 ·002
	10	3 ·021	3 ·021	2 ·006	1 ·001
	9	3 ·050−	2 ·017	1 ·004	1 ·004
	8	2 ·040	1 ·011	0 ·002	0 ·002
	7	1 ·027	0 ·005−	0 ·005−	0 ·005−
	6	0 ·013	0 ·013	—	—
	5	0 ·030	—	—	—
10	13	6 ·024	6 ·024	5 ·007	4 ·002
	12	5 ·035−	4 ·012	3 ·003	3 ·003
	11	4 ·037	3 ·012	2 ·003	2 ·003
	10	3 ·033	2 ·010+	1 ·002	1 ·002
	9	2 ·026	1 ·006	1 ·006	0 ·001
	8	1 ·017	1 ·017	0 ·003	0 ·003
	7	1 ·038	0 ·007	0 ·007	—
	6	0 ·017	0 ·017	—	—
	5	0 ·038	—	—	—
9	13	5 ·017	5 ·017	4 ·005−	4 ·005−
	12	4 ·023	4 ·023	3 ·007	2 ·001
	11	3 ·022	3 ·022	2 ·006	1 ·001
	10	2 ·017	2 ·017	1 ·004	1 ·004
	9	2 ·040	1 ·010+	0 ·001	0 ·001
	8	1 ·025−	1 ·025−	0 ·004	0 ·004
	7	0 ·010+	0 ·010+	—	—
	6	0 ·023	0 ·023	—	—
	5	0 ·049	—	—	—
8	13	5 ·042	4 ·012	3 ·003	3 ·003
	12	4 ·047	3 ·014	2 ·003	2 ·003
	11	3 ·041	2 ·011	1 ·002	1 ·002
	10	2 ·029	1 ·007	1 ·007	0 ·001
	9	1 ·017	1 ·017	0 ·002	0 ·002
	8	1 ·037	0 ·006	0 ·006	—
	7	0 ·015−	0 ·015−	—	—
	6	0 ·032	—	—	—
7	13	4 ·031	3 ·007	3 ·007	2 ·001
	12	3 ·031	2 ·007	2 ·007	1 ·001
A=13 B=7	11	2 ·022	2 ·022	1 ·004	1 ·004
	10	1 ·012	1 ·012	0 ·002	0 ·002
	9	1 ·029	0 ·004	0 ·004	0 ·004
	8	0 ·010+	0 ·010+	—	—
	7	0 ·022	0 ·022	—	—
	6	0 ·044	—	—	—
6	13	3 ·021	3 ·021	2 ·004	2 ·004
	12	2 ·017	2 ·017	1 ·003	1 ·003
	11	2 ·046	1 ·010−	1 ·010−	0 ·001
	10	1 ·024	1 ·024	0 ·003	0 ·003
	9	1 ·050−	0 ·008	0 ·008	—
	8	0 ·017	0 ·017	—	—
	7	0 ·034	—	—	—
5	13	2 ·012	2 ·012	1 ·002	1 ·002
	12	2 ·044	1 ·008	1 ·008	0 ·001
	11	1 ·022	1 ·022	0 ·002	0 ·002
	10	1 ·047	0 ·007	0 ·007	—
	9	0 ·015−	0 ·015−	—	—
	8	0 ·029	—	—	—
4	13	2 ·044	1 ·006	1 ·006	0 ·000
	12	1 ·022	1 ·022	0 ·002	0 ·002
	11	0 ·006	0 ·006	0 ·006	—
	10	0 ·015−	0 ·015−	—	—
	9	0 ·029	—	—	—
3	13	1 ·025	1 ·025	0 ·002	0 ·002
	12	0 ·007	0 ·007	0 ·007	—
	11	0 ·018	0 ·018	—	—
	10	0 ·036	—	—	—
2	13	0 ·010−	0 ·010−	0 ·010−	—
	12	0 ·029	—	—	—
A=14 B=14	14	10 ·049	9 ·020	8 ·008	7 ·003
	13	8 ·038	7 ·016	6 ·006	5 ·002
	12	6 ·023	6 ·023	5 ·009	4 ·003
	11	5 ·027	4 ·011	3 ·004	3 ·004
	10	4 ·028	3 ·011	2 ·003	2 ·003
	9	3 ·027	2 ·009	2 ·009	1 ·002
	8	2 ·023	2 ·023	1 ·006	0 ·001
	7	1 ·016	1 ·016	0 ·003	0 ·003
	6	1 ·038	0 ·008	0 ·008	—
	5	0 ·020	0 ·020	—	—
	4	0 ·049	—	—	—
13	14	9 ·041	8 ·016	7 ·006	6 ·002
	13	7 ·029	6 ·011	5 ·004	5 ·004
	12	6 ·037	5 ·015+	4 ·005+	3 ·002
	11	5 ·041	4 ·017	3 ·006	2 ·001
	10	4 ·041	3 ·016	2 ·005−	2 ·005−
	9	3 ·038	2 ·013	1 ·003	1 ·003
	8	2 ·031	1 ·009	1 ·009	0 ·001

TABLE E.4. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE
AND CORRESPONDING PROBABILITIES (SMALL TYPE) (CONTINUED).

	a	Probability			
		0·05	0·025	0·01	0·005
A = 14 B = 13	7	1 ·021	1 ·021	0 ·004	0 ·004
	6	1 ·048	0 ·010+	—	—
	5	0 ·025−	0 ·025−	—	—
12	14	8 ·033	7 ·012	6 ·004	6 ·004
	13	6 ·021	6 ·021	5 ·007	4 ·002
	12	5 ·025+	4 ·009	4 ·009	3 ·003
	11	4 ·026	3 ·009	3 ·009	2 ·002
	10	3 ·024	3 ·024	2 ·007	1 ·002
	9	2 ·019	2 ·019	1 ·005−	1 ·005−
	8	2 ·042	1 ·012	0 ·002	0 ·002
	7	1 ·028	0 ·005+	0 ·005+	—
	6	0 ·013	0 ·013	—	—
	5	0 ·030	—	—	—
11	14	7 ·026	6 ·009	6 ·009	5 ·003
	13	6 ·039	5 ·014	4 ·004	4 ·004
	12	5 ·043	4 ·016	3 ·005−	3 ·005−
	11	4 ·042	3 ·015−	2 ·004	2 ·004
	10	3 ·036	2 ·011	1 ·003	1 ·003
	9	2 ·027	1 ·007	1 ·007	0 ·001
	8	1 ·017	1 ·017	0 ·003	0 ·003
	7	1 ·038	0 ·007	0 ·007	—
	6	0 ·017	0 ·017	—	—
	5	0 ·038	—	—	—
10	14	6 ·020	6 ·020	5 ·006	4 ·002
	13	5 ·028	4 ·009	4 ·009	3 ·002
	12	4 ·028	3 ·009	3 ·009	2 ·002
	11	3 ·024	3 ·024	2 ·007	1 ·001
	10	2 ·018	2 ·018	1 ·004	1 ·004
	9	2 ·040	1 ·011	0 ·002	0 ·002
	8	1 ·024	1 ·024	0 ·004	0 ·004
	7	0 ·010−	0 ·010−	0 ·010−	—
	6	0 ·022	0 ·022	—	—
	5	0 ·047	—	—	—
9	14	6 ·047	5 ·014	4 ·004	4 ·004
	13	4 ·018	4 ·018	3 ·005−	3 ·005−
	12	3 ·017	3 ·017	2 ·004	2 ·004
	11	3 ·042	2 ·012	1 ·002	1 ·002
	10	2 ·029	1 ·007	1 ·007	0 ·001
	9	1 ·017	1 ·017	0 ·002	0 ·002
	8	1 ·036	0 ·006	0 ·006	—
	7	0 ·014	0 ·014	—	—
	6	0 ·030	—	—	—
8	14	5 ·036	4 ·010−	4 ·010−	3 ·002
	13	4 ·039	3 ·011	2 ·002	2 ·002
	12	3 ·032	2 ·008	2 ·008	1 ·001
	11	2 ·022	2 ·022	1 ·005−	1 ·005−
	10	2 ·048	1 ·012	0 ·002	0 ·002
	9	1 ·026	0 ·004	0 ·004	0 ·004
	8	0 ·009	0 ·009	0 ·009	—
	7	0 ·020	0 ·020	—	—
	6	0 ·040	—	—	—

	a	Probability			
		0·05	0·025	0·01	0·005
A = 14 B = 7	14	4 ·026	3 ·006	3 ·006	2 ·001
	13	3 ·025	2 ·006	2 ·006	1 ·001
	12	2 ·017	2 ·017	1 ·003	1 ·003
	11	2 ·041	1 ·009	1 ·009	0 ·001
	10	1 ·021	1 ·021	0 ·003	0 ·003
	9	1 ·043	0 ·007	0 ·007	—
	8	0 ·015−	0 ·015−	—	—
	7	0 ·030	—	—	—
6	14	3 ·018	3 ·018	2 ·003	2 ·003
	13	2 ·014	2 ·014	1 ·002	1 ·002
	12	2 ·037	1 ·007	1 ·007	0 ·001
	11	1 ·018	1 ·018	0 ·002	0 ·002
	10	1 ·038	0 ·005+	0 ·005+	—
	9	0 ·012	0 ·012	—	—
	8	0 ·024	0 ·024	—	—
	7	0 ·044	—	—	—
5	14	2 ·010+	2 ·010+	1 ·001	1 ·001
	13	2 ·037	1 ·006	1 ·006	0 ·001
	12	1 ·017	1 ·017	0 ·002	0 ·002
	11	1 ·038	0 ·005−	0 ·005−	0 ·005−
	10	0 ·011	0 ·011	—	—
	9	0 ·022	0 ·022	—	—
	8	0 ·040	—	—	—
4	14	2 ·039	1 ·005−	1 ·005−	1 ·005−
	13	1 ·019	1 ·019	0 ·002	0 ·002
	12	1 ·044	0 ·005−	0 ·005−	0 ·005−
	11	0 ·011	0 ·011	—	—
	10	0 ·023	0 ·023	—	—
	9	0 ·041	—	—	—
3	14	1 ·022	1 ·022	0 ·001	0 ·001
	13	0 ·006	0 ·006	0 ·006	—
	12	0 ·015−	0 ·015−	—	—
	11	0 ·029	—	—	—
2	14	0 ·008	0 ·008	0 ·008	—
	13	0 ·025	0 ·025	—	—
	12	0 ·050	—	—	—
A = 15 B = 15	15	11 ·050−	10 ·021	9 ·008	8 ·003
	14	9 ·040	8 ·018	7 ·007	6 ·003
	13	7 ·025+	6 ·010+	5 ·004	5 ·004
	12	6 ·030	5 ·013	4 ·005−	4 ·005−
	11	5 ·033	4 ·013	3 ·005−	3 ·005−
	10	4 ·033	3 ·013	2 ·004	2 ·004
	9	3 ·030	2 ·010+	1 ·003	1 ·003
	8	2 ·025+	1 ·007	1 ·007	0 ·001
	7	1 ·018	1 ·018	0 ·003	0 ·003
	6	1 ·040	0 ·008	0 ·008	—
	5	0 ·021	0 ·021	—	—
	4	0 ·050−	—	—	—

TABLE E.4. SIGNIFICANT LEVELS OF B: VALUES OF B (LARGE TYPE AND CORRESPONDING PROBABILITIES (SMALL TYPE) (CONTINUED).

	a	Probability			
		0·05	0·025	0·01	0·005
A=15 B=14	15	10 ·042	9 ·017	8 ·006	7 ·002
	14	8 ·031	7 ·013	6 ·005⁻	6 ·005⁻
	13	7 ·041	6 ·017	5 ·007	4 ·002
	12	6 ·046	5 ·020	4 ·007	3 ·002
	11	5 ·048	4 ·020	3 ·007	2 ·002
	10	4 ·046	3 ·018	2 ·006	1 ·001
	9	3 ·041	2 ·014	1 ·004	1 ·004
	8	2 ·033	1 ·009	1 ·009	0 ·001
	7	1 ·022	1 ·022	0 ·004	0 ·004
	6	1 ·049	0 ·011	—	—
	5	0 ·025⁺	—	—	—
13	15	9 ·035⁻	8 ·013	7 ·005⁻	7 ·005⁻
	14	7 ·023	7 ·023	6 ·009	5 ·003
	13	6 ·029	5 ·011	4 ·004	4 ·004
	12	5 ·031	4 ·012	3 ·004	3 ·004
	11	4 ·030	3 ·011	2 ·003	2 ·003
	10	3 ·026	2 ·008	2 ·008	1 ·002
	9	2 ·020	2 ·020	1 ·005⁺	0 ·001
	8	2 ·043	1 ·013	0 ·002	0 ·002
	7	1 ·029	0 ·005⁺	0 ·005⁺	—
	6	0 ·013	0 ·013	—	—
	5	0 ·031	—	—	—
12	15	8 ·028	7 ·010⁻	7 ·010⁻	6 ·003
	14	7 ·043	6 ·016	5 [illegible]	4 ·002
	13	6 ·049	5 ·019	4 ·007	3 ·002
	12	5 ·049	4 ·019	3 ·006	2 ·002
	11	4 ·045⁺	3 ·017	2 ·005⁻	2 ·005⁻
	10	3 ·038	2 ·012	1 ·003	1 ·003
	9	2 ·028	1 ·007	1 ·007	0 ·001
	8	1 ·018	1 ·018	0 ·003	0 ·003
	7	1 ·038	0 ·007	0 ·007	—
	6	0 ·017	0 ·017	—	—
	5	0 ·037	—	—	—
11	15	7 ·022	7 ·022	6 ·007	5 ·002
	14	6 ·032	5 ·011	4 ·003	4 ·003
	13	5 ·034	4 ·012	3 ·003	3 ·003
	12	4 ·032	3 ·010⁺	2 ·003	2 ·003
	11	3 ·026	2 ·008	2 ·008	1 ·002
	10	2 ·019	2 ·019	1 ·004	1 ·004
	9	2 ·040	1 ·011	0 ·002	0 ·002
	8	1 ·024	1 ·024	0 ·004	0 ·004
	7	1 ·049	0 ·010⁻	0 ·010⁻	—
	6	0 ·022	0 ·022	—	—
	5	0 ·046	—	—	—
10	15	6 ·017	6 ·017	5 ·005⁻	5 ·005⁻
	14	5 ·023	5 ·023	4 ·007	3 ·002
	13	4 ·022	4 ·022	3 ·007	2 ·001
	12	3 ·018	3 ·018	2 ·005⁻	2 ·005⁻
	11	3 ·042	2 ·013	1 ·003	1 ·003
	10	2 ·029	1 ·007	1 ·007	0 ·001
	9	1 ·016	1 ·016	0 ·002	0 ·002
	8	1 ·034	0 ·006	0 ·006	—
	7	0 ·013	0 ·013	—	—
	6	0 ·028	—	—	—
9	15	6 ·042	5 ·012	4 ·003	4 ·003
	14	5 ·047	4 ·015⁻	3 ·004	3 ·004
A=15 B=9	13	4 ·042	3 ·013	2 ·003	2 ·003
	12	3 ·032	2 ·009	2 ·009	1 ·002
	11	2 ·021	2 ·021	1 ·005⁻	1 ·005⁻
	10	2 ·045⁻	1 ·011	0 ·002	0 ·002
	9	1 ·024	1 ·024	0 ·004	0 ·004
	8	1 ·048	0 ·009	0 ·009	—
	7	0 ·019	0 ·019	—	—
	6	0 ·037	—	—	—
8	15	5 ·032	4 ·008	4 ·008	3 ·002
	14	4 ·033	3 ·009	3 ·009	2 ·002
	13	3 ·026	2 ·006	2 ·006	1 ·001
	12	2 ·017	2 ·017	1 ·003	1 ·003
	11	2 ·037	1 ·008	1 ·008	0 ·001
	10	1 ·019	1 ·019	0 ·003	0 ·003
	9	1 ·038	0 ·006	0 ·006	—
	8	0 ·013	0 ·013	—	—
	7	0 ·026	—	—	—
	6	0 ·050⁻	—	—	—
7	15	4 ·023	4 ·023	3 ·005⁻	3 ·005⁻
	14	3 ·021	3 ·021	2 ·004	2 ·004
	13	2 ·014	2 ·014	1 ·002	1 ·002
	12	2 ·032	1 ·007	1 ·007	0 ·001
	11	1 ·015⁺	1 ·015⁺	0 ·002	0 ·002
	10	1 ·032	0 ·005⁻	0 ·005⁻	0 ·005⁻
	9	0 ·010⁺	0 ·010⁺	—	—
	8	0 ·020	0 ·020	—	—
	7	0 ·038	—	—	—
6	15	3 ·015⁺	3 ·015⁺	2 ·003	2 ·003
	14	2 ·011	2 ·011	1 ·002	1 ·002
	13	2 ·031	1 ·006	1 ·006	0 ·001
	12	1 ·014	1 ·014	0 ·002	0 ·002
	11	1 ·029	0 ·004	0 ·004	0 ·004
	10	0 ·009	0 ·009	0 ·009	—
	9	0 ·017	0 ·017	—	—
	8	0 ·032	—	—	—
5	15	2 ·009	2 ·009	2 ·009	1 ·001
	14	2 ·032	1 ·005⁻	1 ·005⁻	1 ·005⁻
	13	1 ·014	1 ·014	0 ·001	0 ·001
	12	1 ·031	0 ·004	0 ·004	0 ·004
	11	0 ·008	0 ·008	0 ·008	—
	10	0 ·016	0 ·016	—	—
	9	0 ·030	—	—	—
4	15	2 ·035⁺	1 ·004	1 ·004	1 ·004
	14	1 ·016	1 ·016	0 ·001	0 ·001
	13	1 ·037	0 ·004	0 ·004	0 ·004
	12	0 ·009	0 ·009	0 ·009	—
	11	0 ·018	0 ·018	—	—
	10	0 ·033	—	—	—
3	15	1 ·020	1 ·020	0 ·001	0 ·001
	14	0 ·005⁻	0 ·005⁻	0 ·005⁻	0 ·005⁻
	13	0 ·012	0 ·012	—	—
	12	0 ·025⁻	0 ·025⁻	—	—
	11	0 ·043	—	—	—
2	15	0 ·007	0 ·007	0 ·007	—
	14	0 ·022	0 ·022	—	—
	13	0 ·044	—	—	—

APPENDIX F

PROBIT ANALYSIS[1]

1. PROBIT ANALYSIS OF FATHEAD MINNOW EMBRYO-LARVAL DATA

1.1 This program calculates the LC1 value and associated 95% confidence limits using Probit Analysis. The program, written in FORTRAN PLUS IV v.3, was adapted by James Dryer, EMSL-Cincinnati, from a program obtained from Drs. Wesley Birge and Jeffrey Black, Graduate Center for Toxicology, University of Kentucky, Lexington.

1.2 The program runs on the Digital Equipment Corporation, PDP-11/70 computer under IAS version 3.1. Machine readable copies of the program and assistance in running it can be obtained by contacting James Dryer, EMSL-Cincinnati Newtown Facility (FTS 778-8350; comm'l 513-527-8350).

1.3 Sample Data Input and Output from the Computer Program

1.3.1 The data input process and the output provided by the computer program are illustrated with a set of mortality data from a fathead minnow embryo-larval survival and teratogenicity test.

The program begins with a request for the following information:

1. The number of concentrations.
2. The number of test organisms exposured in the control.
3. The number of test organisms that died in the control.

The program then requests information on each toxicant concentration:

1. The concentration value.
2. The total number that died at each toxicant concentration.
3. The number of test organisms exposed at each toxicant concentration.

Probit statistics are then listed for the analysis, including a table which provides the LC1 value and confidence limits.

---

[1] Prepared by James Dryer, Aquatic Biology Section, EMSL-Cincinnati Newtown Facility, U. S. Environmental Protection Agency, 3411 Church Street, Newtown, Ohio

1.3.2 Sample Data Input and Output from the Computer Program for Probit Analysis of Fathead Minnow Embryo-larval Data

```
  PROBIT ANALYSIS***MAXIMUM LIKELIHOOD SOLUTION

        USED FOR CALCULATING LC1 S, . . . . LC99 S

   MAXIMUM NUMBER OF POINTS IS 20.

 NUMBER OF CONCENTRATIONS = 5
 CONTROL SAMPLE SIZE = 100.
 NUMBER OF DEAD IN THE CONTROL GROUP17.
  BEGIN INPUTTING OF DATA WITH LOWEST CONCENTRATION

 CONCENTRATION = 3.
 NUMBER RESPONDING (DEAD) = 14.
 NUMBER TREATED = 100.

 CONCENTRATION = 5.
 NUMBER RESPONDING (DEAD) = 16.
 NUMBER TREATED = 102.

 CONCENTRATION = 7.
 NUMBER RESPONDING (DEAD) = 35.
 NUMBER TREATED = 100.

 CONCENTRATION = 11.
 NUMBER RESPONDING (DEAD) = 72.
 NUMBER TREATED = 99.

 CONCENTRATION = 16.
 NUMBER RESPONDING (DEAD) = 99.
 NUMBER TREATED = 99.

 INPUT CONCENTRATION SCALE IS TRANSFORMED TO LOG(10)
```

1.3.2 Sample data input and output from the computer program (continued).

```
-----------------------------------------------------------------------------

                                P R O B I T   T A B L E
    LOG
CONCENTRATION SAMPLE         OBSERVED        EXPECTED       DEVIATION    PROBABILITY

   0.4771        100.           14.           15.58          -1.58         0.1558
   0.6990        102.           16.           17.79          -1.79         0.1744
   0.8451        100.           35.           31.32           3.68         0.3132
   1.0414         99.           72.           76.43          -4.43         0.7720
   1.2041         99.           99.           96.37           2.63         0.9734

  CONTROL        100.           17.           15.57           1.43         0.1557
-----------------------------------------------------------------------------

THF CONSTANTS USED IN THIS PROBLEM WERE;

     HETEROGENEITY FACTOR =   1.5886
         NUMBER OF POINTS =   5
       DEGREES OF FREEDOM =   3
                  DEVIATE =   1.9600
                        G =   0.0515
THE TOTAL NUMBER OF CYCLES=   2.

THE STATISTICS ARE;
                    AVG Y =      5.3697
                    AVG X =      1.0059
                    AVG T =      1.6983
        NATURAL MORTALITY =      0.1557    SE =     0.0229
                    SLOPE =      8.0356    SE =     0.9306
                INTERCEPT =     -2.7134
              CHI SQUARED =      4.7658

-----------------------------------------------------------------------------
    POINT      CONCENTRATION              LOWER                   UPPER
                                            95% CONFIDENCE LIMITS

  LC1               4.6817                3.6430                  5.5067
-----------------------------------------------------------------------------

NOTE:IF THE LC1 IS ABOVE/BELOW THE HIGHEST/LOWEST CONCEN-
TRATION  TESTED, EXTREME CAUTION  SHOULD BE EXERCISED IN
REGARD TO  USING SUCH VALUE BECAUSE IT LIES  OUTSIDE THE
KNOWN RANGE AND IS ONLY AN EXTRAPOLATED ESTIMATED  VALUE.
SIMILAR  CAUTIONS  SHOULD  ALSO BE USED IN REGARD TO THE
UPPER  AND  LOWER 95% CONFIDENCE LIMITS FOR LIKE REASONS.
FOR FURTHER CLARIFICATION, PLEASE CONSULT YOUR QUALIFIED
STATISTICIAN.
```

1.3.3 Listing of Computer Program for Probit Analysis of Fathead Minnow Embryo-larval Data

---

```
      IMPLICIT REAL*8 (A-H,O-Z)
      DIMENSION FMT(13),SG(20),SAVER(20),SAVEN(20),VEC(13)
      DIMENSION EXPY(20),A998(20),A999(20),X14(20),TO5(18)
      DIMENSION BIGP(20),CHISQ(18),OBSY(20),ENP(20)
      DIMENSION X(20),EN(20),R(20),SMALP(20)
      COMMON OBSY,X,A,BNEW,BIGC,N,ILOG
      DIMENSION OP(1),OY(1)
      DATA OP,OY/0.01,2.6737/
C
C     T AND CHISQ VALUES STORED.
C
C  NOTE; THIS VERSION WRITTEN 9/16/85.
C
      DATA TO5,CHISQ/12.706,4.303,3.182,2.776,2.571,
     * 2.447,2.365,2.306,2.262,2.228,2.201,2.179,2.160,
     * 2.145,2.131,2.120,2.110,2.101,3.841,5.991,7.815,
     * 9.488,11.07,12.592,14.067,15.507,16.919,18.307,
     * 19.675,21.026,22.362,23.685,24.996,26.296,27.587,
     * 28.869/
      MIN=5
  100 TYPE*,' '
      TYPE*,' PROBIT ANALYSIS***MAXIMUM LIKELIHOOD SOLUTION'
      TYPE*,' '
      TYPE*,'      USED FOR CALCULATING THE LC1'
      TYPE*,' '
      TYPE*,'  MAXIMUM NUMBER OF POINTS IS 20.'
      TYPE*,' '
      TYPE 2
    2 FORMAT(' NUMBER OF CONCENTRATIONS = ',$)
      ACCEPT 3,N
    3 FORMAT(I2)
      TYPE 4
    4 FORMAT(' CONTROL SAMPLE SIZE = ',$)
   10 FORMAT(F3.0)
      ACCEPT 10,CNTRL
      IF(CNTRL.GT.0)GO TO 1
    1 TYPE 5
    5 FORMAT (' NUMBER OF DEAD IN THE CONTROL GROUP = ',$)
      ACCEPT 10,DEAD
      IND=0
      GO TO 200
  192 GO TO 9999
  200 ILOG = 1
      GO TO 260
  230 DO 250 J = 1, N
      EN(J) = SAVEN(J)
      R(J) = SAVER(J)
  250 X(J) = DLOG10(X(J))
      IND=2
      ILOG = 2
  260 ICYCL=-1
      B=0.D0
      K=N-2
      IF(CNTRL)280,280,270
  270 SMALC=DEAD/CNTRL
      BIGC=SMALC+0.0000001D0
      GO TO 290
  280 SMALC=(-1.D0)
      BIGC=0.D0
```

1.3.3 Listing of Program for Probit Analysis of Fathead Minnow Embryo-larval Data (Continued)

```
  290 GO TO (300,320), ILOG
  300 TYPE*,' '
      TYPE*,' ==> BEGIN INPUTTING OF DATA WITH THE LOWEST CONCENTRATION'
      TYPE*,' '
      DO 310 J=1,N
      TYPE 311
  311 FORMAT(' CONCENTRATION = ',$)
      ACCEPT 312,X(J)
  312 FORMAT(F35.13)
      X14(J)=X(J)
      TYPE 313
  313 FORMAT(' NUMBER RESPONDING (DEAD) = ',$)
      ACCEPT 314,R(J)
  314 FORMAT(F6.2)
      TYPE 316
  316 FORMAT(' NUMBER TREATED = ',$)
      ACCEPT 314,EN(J)
      TYPE*,' '
      SAVEN(J)=EN(J)
      SAVER(J)=R(J)
  310 CONTINUE
C
C     COMPUTE FIRST CYCLE OF PROBITS WITHOUT AUXILIARY VARIATE
C     IF ICYCL = -1.
C     IF ICYCL EXCEEDS -1 EXP. PROBITS BECOME PROVISIONAL PROBITS.
C
      IF((CNTRL.LE.0  ).OR.(ICYCL.GE.0))  GO TO 320
      ANUM=0.0D0
      DENOM=0.0D0
      DO 315 I=1,N
      IF(R(I)/EN(I).GE.2.D0*SMALC) GO TO 315
      ANUM=ANUM+R(I)
      DENOM=DENOM+EN(I)
  315 CONTINUE
      IF(DENOM.EQ.0) GO TO 320
      BIGC=(ANUM+DEAD)/(DENOM+CNTRL)
  320 IF(IND.LT.1) GO TO 230
      DO 400 I=1,N
      SMALP(I)=(R(I)/EN(I)-BIGC)/(1.D0-BIGC)
    6 FORMAT(I3)
      IF(ICYCL)350,390,390
  350 IF(SMALP(I).LE.0.D0) GO TO 330
      IF(SMALP(I).GE.1.D0) GO TO 340
      GO TO 380
  330 SMALP(I)=.0001D0
      GO TO 380
  340 SMALP(I)=.9999D0
  380 OBSY(I)=PROBIT(SMALP(I))
      GO TO 400
  390 OBSY(I)=EXPY(I)
  400 CONTINUE
C
C     COMPUTE STATISTICS
C
C
      SWN=0.D0
      SWNX=0.D0
      SWNY=0.D0
      SWNXX=0.D0
      SWNXY=0.D0
      SWNYY=0.D0
```

1.3.3 Listing of Program for Probit Analysis of Fathead Minnow Embryo-larval Data (Continued)

```
    SWNT=0.D0
    SWNXT=0.D0
    SWNTT=0.D0
    SWNTY=0.D0
    DO 470 I=1,N
    ED=OBSY(I)-5.D0
    IF(DABS(ED)-18.D0) 401,401,402
401 Z=.39894220401433D0*DEXP(-(ED*ED*.5D0))
    GO TO 403
402 Z=.1D-15
403 IF (ICYCL) 410,420,420
410 BIGP (I)=SMALP(I)
    GO TO 440
420 AH=DABS(ED*DSQRT(.5D0))
    IF(AH.LT.30.D0)  GO TO 430
    AI=.5D0
    GO TO 431
430 AI=((0.0000430638D0*AH+0.0002765672D0)*AH+0.0001520143D0)*AH+0.009
   12705272D0
    AI=.5D0-.5D0/(((AI*AH+.0422820123D0)*AH+.0705230784D0)*AH+1.D0)**1
   16
431 BIGP(I)=.5D0+DSIGN(AI,ED)
440 EXPY(I)=OBSY(I)+(SMALP(I)-BIGP(I))/Z
    WNP=EN(I)/(BIGP(I)+BIGC/(1.D0-BIGC))
    TP=(1.D0-BIGP(I))
    IF((BIGP(I).LT.1.D0).AND.(DABS(ED).LE.13.D0))  GO TO 441
    WN=0.D0
    GO TO 442
441 WN=WNP*Z*Z/TP
442 EX=X(I)
    EXWN=EX*WN
    PRBT=EXPY(I)
    SWN = SWN + WN
    YWN=PRBT*WN
    SWNYY = SWNYY + PRBT * YWN
    SWNX=SWNX+EXWN
    SWNY=SWNY+YWN
    SWNXX=SWNXX+EX*EXWN
    SWNXY=SWNXY+EX*YWN
    IF (ICYCL) 470,450,450
450 IF(SMALC)470,460,460
460 CONTINUE
    TWN=WNP*Z
    SWNT=SWNT+TWN
    SWNXT=SWNXT+EX*TWN
    SWNTT=SWNTT+WNP*TP
    SWNTY=SWNTY+TWN*PRBT
470 CONTINUE
    IF(SWN.EQ.0.D0) GO TO 520
    AVGX=SWNX/SWN
    AVGY=SWNY/SWN
    AVGT=SWNT/SWN
    SWNXX=SWNXX-SWNX*AVGX
    SWNXY=SWNXY-SWNX*AVGY
    SWNYY=SWNYY-SWNY*AVGY
    IF((SWNXX.EQ.0).OR.(SWNXY.EQ.0)) GO TO 520
    BNEW=SWNXY/SWNXX
    BB=BNEW
    A=AVGY-BNEW*AVGX
    IF(SMALC) 471,472,472
471 REGSS=BNEW*SWNXY
```

1.3.3 Listing of Program for Probit Analysis of Fathead Minnow Embryo-larval Data (Continued)

```
      C11=1.D0/SWNXX
      C12=0.D0
      C22=0.D0
  472 IF (ICYCL) 480,500,500
  480 DO 490 I=1,N
  490 EXPY(I)=A+BNEW*X(I)
      B=BNEW
      ICYCL=ICYCL+1
      GO TO 320
  500 IF(SMALC) 520,510,510
  510 SWNXT=SWNXT-SWNX*AVGT
      CNST=SMALC-BIGC
      SWNTY=SWNTY-SWNT*AVGY+((CNST/BIGC)*CNTRL)
      SWNTT=SWNTT-SWNT*AVGT+(1.D0-BIGC)/BIGC*CNTRL
      SWNYY=SWNYY+CNTRL*CNST/BIGC*CNST/(1.D0-BIGC)
      C11=1.D0/(SWNXX-SWNXT/SWNTT*SWNXT)
      C12=1.D0/(SWNXT-SWNTT/SWNXT*SWNXX)
      C22=1.D0/(SWNTT-SWNXT/SWNXX*SWNXT)
      BNEW=C11*SWNXY+C12*SWNTY
  512 DOOMC=C12*SWNXY+C22*SWNTY
      A=AVGY-BNEW*AVGX-DOOMC*AVGT
      BIGC=BIGC+DOOMC*(1.D0-BIGC)
      REGSS=BNEW*SWNXY+DOOMC*SWNTY
  520 DO 530 I=1,N
  530 EXPY(I)=A+BNEW*X(I)
      ICYCL=ICYCL+1
      TEST=BNEW-B
      XSQ=SWNYY-REGSS
      IF(TEST)540,550,550
  540 TEST=-TEST
  550 IF(ICYCL-100)560,580,580
  560 IF(SMALC)561,565,565
  561 IF(TEST.LE.00001D0)  GO TO 610
      GO TO 570
  565 IF((TEST.LE..01D0).OR.(DABS(BNEW-BB).LT..005D0)) GO TO 610
  570 B=BNEW
      GO TO 320
  580 TEST=BNEW-B
  590 WRITE(5,600)TEST
  600 FORMAT(49H 100 CYCLES HAVE BEEN COMPLETED. B(100) - B(99) =,F14.7)
C
C
C     CALCULATE CHISQ AND TEST FOR HETEROGENEITY.
C
  610 L=0
      IF(K.EQ.0) GO TO 820
      IF ( XSQ.GE.CHISQ(K)) GO TO 650
      U=1.96
  645 GO TO 820
  650 WRITE(5,660) XSQ
      U=T05(K)
  660 FORMAT (47H CHI SQUARED ESTIMATE OBTAINED BY SUBTRACTION =,F10.5)
C
C
  670 DO 680 I=1,N
C     SET UP EXPECTED NUMBERS.
  680 ENP(I)=EN(I)*(BIGP(I)*(1.D0-BIGC)+BIGC)
  820 FK=K
      IF(K.EQ.0) GO TO 850
      HET=XSQ/FK
      FEXP=T05(K)*T05(K)
```

1.3.3 Listing of Program for Probit Analysis of Fathead Minnow Embryo-larval Data (Continued)

```
      FCAL=REGSS/HET
C     TEST REGRESSION SS.IF SIGNIFICANT
C     SET UP CONSTANTS AND COMPUTE STATISTICS.
      IF(FCAL-FEXP)830,830,850
  830 WRITE(5,840)
  840  FORMAT(  26H NONSIGNIFICANT REGRESSION/)
  850 G=U*U*C11/BNEW/BNEW
      DO 920 J=1,N
      EXPY(J)=BIGP(J)*(1.D0-BIGC)+BIGC
      ENP(J)=SAVEN(J)*EXPY(J)
      OBSY(J)=(SAVER(J)/SAVEN(J)-BIGC)/(1.D0-BIGC)
      IF(OBSY(J).LE.0.D0) GO TO 860
      IF(OBSY(J).GE.1.D0) GO TO 870
      OBSY(J)=PROBIT(OBSY(J))
      GO TO 920
  860 OBSY(J)=1.D0
      GO TO 920
  870 OBSY(J)=8.D0
  920 BIGP(J)=SAVER(J)-ENP(J)
      TYPE*,'-----------------------------------------------------------
     1-----------------------'
      TYPE*,' '
      TYPE*,' '
      TYPE*,'                              P R O B I T   T A B L E'
      IF(ILOG.EQ.2) WRITE(5,929)
  929 FORMAT(6X,3HLOG)
      WRITE(5,930) (X(J),SAVEN(J),SAVER(J),ENP(J),SG(J),BIGP(J),
     1  EXPY(J),J=1,N)
  930 FORMAT(1X,13HCONCENTRATION,1X,6HSAMPLE,6X,8HOBSERVED,6X,8HEXPECTE
     1D ,7X,9HDEVIATION,3X,11HPROBABILITY//(1X,F8.4,6X,F6.0,8X,F6.0,
     26X,F8.2,A2,6X,F8.2,4X,F10.4))
      SEB=DSQRT(C11)
      SEC=(1.D0-BIGC)*DSQRT(C22)
      IF(SMALC.LT.0.D0) GO TO 940
      TEST=CNTRL*BIGC
      B=DEAD-TEST
      WRITE(5,935)CNTRL,DEAD,TEST,B,BIGC
      TYPE*,'-------------------------------------------------------------
     1---------------------'
  935 FORMAT(9H0 CONTROL,6X,F6.0,8X,F6.0,6X,F8.2,6X,F8.2,4X,F10.4)
      WRITE(5,950)
  950 FORMAT(41H0THE CONSTANTS USED IN THIS PROBLEM WERE;/)
      WRITE(5,960)HET
  960 FORMAT(6X,22HHETEROGENEITY FACTOR =,F9.4)
      WRITE(5,970)N
  970 FORMAT(10X,19HNUMBER OF POINTS = ,I3)
      WRITE(5,980)K
  980 FORMAT(8X,21HDEGREES OF FREEDOM = ,I3)
      WRITE(5,990)U
  990 FORMAT(19X,9HDEVIATE =,F9.4)
      WRITE(5,1000)G
 1000 FORMAT(25X,3HG =,F9.4)
      WRITE(5,1010)ICYCL
      TYPE*,' '
 1010 FORMAT(28H THE TOTAL NUMBER OF CYCLES=,I4,1H.)
  940 IF(L.GT.0) WRITE(5,945)
  945 FORMAT(1H0,48X,'*=EXPECTED LESS THAN 5'/49X,'**=EXPECTED MORE THA
     1N N-5')
      WRITE(5,1020)
 1020 FORMAT(22H0THE STATISTICS ARE;    )
      WRITE(5,1030)AVGY
```

1.3.3 Listing of Program for Probit Analysis of Fathead Minnow Embryo-larval Data (Continued)

```
 1030 FORMAT(21X,7HAVG Y =,F12.4)
      WRITE(5,1040)AVGX
 1040 FORMAT(21X,7HAVG X = ,F12.4)
      WRITE(5,1050)AVGT
 1050 FORMAT(21X,7HAVG T = ,F12.4)
      WRITE(5,1060)BIGC,SEC
 1060 FORMAT(9X,19HNATURAL MORTALITY =,F12.4,4X,4HSE = ,F10.4)
      WRITE(5,1070)BNEW,SEB
 1070 FORMAT(21X,7HSLOPE = ,F12.4,4X,4HSE =,F10.4)
      WRITE(5,1080)A
 1080 FORMAT(17X,11HINTERCEPT =,F12.4)
      WRITE(5,1090)XSQ
 1090 FORMAT(15X,13HCHI SQUARED =,F12.4/)
      IF(XSQ.GE.CHISQ(K))WRITE(5,1095)
 1095 FORMAT(  '0*CHI SQUARED IS SIGNIFICANT.  HETEROGENEITY FACTOR IS
     1  NOT USED.  USE EXTREME CAUTION*'/)
      TYPE*,'____________________________________________________________
     1_____________________'
 1105 WRITE(5,1100)
 1100 FORMAT(5X,5HPOINT,6X,13HCONCENTRATION,12X,5HLOWER ,16X,5HUPPEP)
C
      WRITE(5,987)
  987 FORMAT('                                                95% CONFID
     1ENCE LIMITS')
      TYPE*,' '
C     COMPUTE FIDUCIAL LIMITS.
      ONE=G/(1.D0-G)
      THREE=U/(BNEW*(1.D0-G))
      FIVE=G*(1.D0/SWN+AVGT*AVGT*(C22-C12*C12/C11))
      EM=(OY(1)-A)/BNEW
      IF(THREE.LE.0.D0) GO TO 1150
 1110 EMLSX=EM-AVGX
      TWO=EMLSX-AVGT*C12/C11
      FOUR=1.D0/SWN+AVGT*AVGT*C22-2.D0*EMLSX*AVGT*C12+EMLSX*EMLSX*C11
      PART2=EM+ONE*TWO
      PART3=THREE*DSQRT(FOUR-FIVE)
      XLL=PART2-PART3
      XUL=PART2+PART3
      GO TO (1130,1120),ILOG
 1120 EM=DEXP(2.302585D0*EM)
      XLL=DEXP(2.302585D0*XLL)
      XUL=DEXP(2.302585D0*XUL)
 1130 IF(EM.GT.X14(N))A999(J)=OP(1)*100
      IF(EM.LT.X14(1))A998(J)=OP(1)*100
      WRITE(5,1140) EM,XLL,XUL
 1140 FORMAT(5H  LC1,4X,'   ',F16.4,5X,F16.4,3X,F16.4)
      GO TO 1190
 1150 GO TO (1170,1160),ILOG
 1160 EM=DEXP(2.302585D0*EM)
 1170 WRITE(5,1180) OP(1)*100.,EM
 1180 FORMAT(' LC',F3.0,12X,F16.4,8X,' U N C A L C U L A B L E')
 1190 TYPE*,'------------------------------------------------------------
     1-------------------'
      TYPE*,' '
      TYPE*,' '
      TYPE*,'NOTE:IF THE LC1 IS ABOVE/BELOW THE HIGHEST/LOWEST CONCEN-'
      TYPE*,'TRATION  TESTED, EXTREME CAUTION  SHOULD BE EXERCISED IN'
      TYPE*,'REGARD TO  USING SUCH VALUE BECAUSE IT LIES  OUTSIDE THE'
      TYPE*,'KNOWN RANGE AND IS ONLY AN EXTRAPOLATED ESTIMATED  VALUE.'
      TYPE*,'SIMILAR  CAUTIONS  SHOULD  ALSO BE USED IN REGARD TO THE'
      TYPE*,'UPPER  AND  LOWER 95% CONFIDENCE LIMITS FOR LIKE REASONS.'
```

1.3.3 Listing of Program for Probit Analysis of Fathead Minnow Embryo-larval Data (Continued)

```
      TYPE*,'FOR FURTHER CLARIFICATION, PLEASE CONSULT YOUR QUALIFIED'
      TYPE*,'STATISTICIAN.'
      TYPE*,' '
      TYPE*,' '
      WRITE(5,9991)
 9991 FORMAT(//////////////////////////)
 9999 STOP
      END
      REAL FUNCTION PROBIT*8(PROP)
      IMPLICIT REAL*8 (A-H,O-Z)
      TEMP=DMIN1(PROP,1.-PROP)
      TEMP=DSQRT(-2.D0*DLOG(TEMP))
      TEMP=TEMP- (2.515517D0+TEMP*(0.802853D0+TEMP*0.010328D0))/
     1  (1.D0+TEMP*(1.432788D0+TEMP*(0.189269D0+TEMP*0.001308D0)))
      PROBIT=5.D0+DSIGN(TEMP,(PROP-0.5D0))
      RETURN
      END
```

2. PROBIT ANALYSIS OF ALGAL GROWTH TEST DATA

2.1 This program calculates the EC1 value and associated 95% confidence limits using Probit Analysis. The program, written in FORTRAN PLUS IV v.3, was adapted by James Dryer, EMSL-Cincinnati, from a program obtained from Drs. Wesley Birge and Jeffrey Black, Graduate Center for Toxicology, University of Kentucky, Lexington.

2.2 The program runs on the Digital Equipment Corporation, PDP-11/70 computer under IAS version 3.1. Machine readable copies of the program and assistance in running it can be obtained by contacting James Dryer, EMSL-Cincinnati Newtown Facility (FTS 778-8350; comm'l 513-527-8350).

2.3 Sample Data Input and Output from the Computer Program

2.3.1 The cell counts are input to the program. (Counts obtained with the Coulter Counter, must first be "corrected" for coincidence.). The program determines the mean count for each concentration, divides each of the treatment means by the control mean, and subtracts the quotient from 1.0 to form an inhibition proportion for each concentration. The proportions are arbitrarily based on a population size of 100.

2.3.2 Sample Data Input And Output From The Computer Program for Probit Analysis of Algal Growth Test Data

```
PROBIT ANALYSIS-USED FOR CALCULATING THE EC1 (ALGAL TEST)

EXCLUDING CONTROL, NUMBER OF CONCENTRATIONS = 5

NUMBER OF VALUES FOR CONTROL  = 3

PLEASE ENTER THE  3 CORRECTED COUNTS IN CONTROL.

CONTROL COUNT = 1209.
CONTROL COUNT = 1180.
CONTROL COUNT = 1340.

   BEGIN INPUTTING OF DATA WITH LOWEST CONCENTRATION

CONCENTRATION = 5.
PLEASE ENTER THE  3 CORRECTED COUNTS IN CONC   5.00
COUNTS =1212.
COUNTS =1186.
COUNTS =1204.

CONCENTRATION = 10.
PLEASE ENTER THE  3 CORRECTED COUNTS IN CONC  10.00
COUNTS =826.
COUNTS =628.
COUNTS =816.

CONCENTRATION = 20.
PLEASE ENTER THE  3 CORRECTED COUNTS IN CONC  20.00
COUNTS =493.
COUNTS =416.
COUNTS =413.

CONCENTRATION = 40.
PLEASE ENTER THE  3 CORRECTED COUNTS IN CONC  40.00
COUNTS =127.
COUNTS =147.
COUNTS =147.

CONCENTRATION = 80.
PLEASE ENTER THE  3 CORRECTED COUNTS IN CONC, 80.00
COUNTS =49.3
COUNTS =40.
COUNTS =44.
```

2.3.2 Sample Data Input And Output From The Computer Program for Probit Analysis of Algal Growth Test Data (Continued)

```
NOTE; CHI SQUARED ESTIMATE OBTAINED BY SUBTRACTION
-----------------------------------------------------------------


               P R O B I T   T A B L E
CONCENTRATION  PERCENT         PROBABILITY

  5.0000         3.41            0.0881
 10.0000        39.13            0.3090
 20.0000        64.55            0.6387
 40.0000        88.71            0.8866
 80.0000        96.43            0.9804

-----------------------------------------------------------------

THE CONSTANTS USED IN THIS PROBLEM WERE;

     HETEROGENEITY FACTOR =    2.7298
         NUMBER OF POINTS =    5
       DEGREES OF FREEDOM =    3
                  DEVIATE =    3.1820
         NUMBER OF CYCLES =    9.

THE STATISTICS ARE;
                    AVG Y =       5.0917
                    AVG X =       1.2082
                    AVG T =       1.9104
                    SLOPE =       2.8363     SE =      0.2100
                INTERCEPT =       1.6650
                        G =       0.0555
              CHI SQUARED =       8.1895

--------------------------------------------------------------------------------
    POINT      CONCENTRATION                LOWER                    UPPER
                                              95% CONFIDENCE LIMITS

    EC1             2.2681                   1.1959                   3.4151
--------------------------------------------------------------------------------


NOTE: IF THE EC1  VALUE IS ABOVE/BELOW THE HIGHEST/LOWEST
CONCENTRATION TESTED, EXTREME CAUTION SHOULD BE EXERCISED
IN REGARD TO SUCH VALUE BECAUSE IT LIES OUTSIDE THE KNOWN
RANGE AND IS ONLY AN EXTRAPOLATED ESTIMATED VALUE.SIMILAR
CAUTIONS  SHOULD ALSO BE  USED IN REGARD TO THE UPPER AND
LOWER 95% CONFIDENCE LIMITS FOR LIKE REASONS. SHOULD FUR-
THER ASSISTANCE BE REQUIRED,PLEASE CONSULT YOUR QUALIFIED
STATISTICIAN.
```

2.3.3 Listing Of Computer Program For Probit Analysis Of Algal Growth Test Data

```
      IMPLICIT REAL*8 (A-H,O-Z)
      DIMENSION FMT(13),SAVER(20),SAVEN(20),VEC(13)
      DIMENSION EXPY(20),A998(20),A999(20),X14(20),T05(18)
      DIMENSION BIGP(20),CHISQ(18),OBSY(20),ENP(20)
      DIMENSION X(20),R7(20),R(20),SMALP(20)
      DIMENSION SG(20),R1(20),P5(20),CONCNT(20)
      COMMON OBSY,X,A,BNEW,BIGC,N,ILOG
C
C     T AND CHISQ VALUES STORED.
C
C   N O T E ;   T H I S   V E R S I O N   W R I T T E N   9 / 1 6 / 85
C
C
      DATA T05,CHISQ/12.706,4.303,3.182,2.776,2.571,
     * 2.447,2.365,2.306,2.262,2.228,2.201,2.179,2.160,
     * 2.145,2.131,2.120,2.110,2.101,3.841,5.991,7.815,
     * 9.488,11.07,12.592,14.067,15.507,16.919,18.307,
     * 19.675,21.026,22.362,23.685,24.996,26.296,27.587,
     * 28.869/
      MIN=5
  100 TYPE*,' '
      TYPE*,'PROBIT ANALYSIS-USED FOR CALCULATING THE EC1 (ALGAL TEST)'
      TYPE*,' '
      TYPE 2
    2 FORMAT(' EXCLUDING CONTROL, NUMBER OF CONCENTRATIONS = ',$)
      ACCEPT 3,N
    3 FORMAT(I2)
      TYPE*,' '
      TYPE 5
    5 FORMAT(' NUMBER OF VALUES FOR CONTROL  = ',$)
      ACCEPT 6,K1
    6 FORMAT(I3)
      TYPE*,' '
      WRITE(5,1)K1
    1 FORMAT(' PLEASE ENTER THE',I3,' CORRECTED COUNTS IN CONTROL.')
      TYPE*,' '
      DO 10 J=1,K1
      TYPE 501
  501 FORMAT(' CONTROL COUNT = ',$)
      ACCEPT 521,CONCNT(J)
      CNT=CNT+CONCNT(J)
   10 CONTINUE
      TYPE*,' '
      CNT=CNT/K1
      IND=0
  200 ILOG = 1
      GO TO 260
  230 TYPE*,' '
      IND=2
      ILOG = 2
  260 ICYCL=-1
      B=0.
      K=N-2
      SMALC=0.
      BIGC=.001
  521 FORMAT(F12.4)
      GO TO(300,320),ILOG
  300 TYPE*,'   BEGIN INPUTTING OF DATA WITH LOWEST CONCENTRATION'
      TYPE*,' '
```

2.3.3 Listing of Computer Program for Probit Analysis of Algal Growth Test Data (Continued)

```
      DO 310 J=1,N
      TYPE 311
  311 FORMAT(' CONCENTRATION = ',$)
      ACCEPT 312,X(J)
  312 FORMAT(F35.13)
      X14(J)=X(J)
      X(J)=LOG10(X(J))
      WRITE(5,314)K1,X14(J)
  314 FORMAT(' PLEASE ENTER THE',I3,' CORRECTED COUNTS IN CONC',F7.2)
      DO 313 K=1,K1
      TYPE 316
  316 FORMAT(' COUNTS =',$)
      ACCEPT 312,R7(K)
      R1(J)=R1(J)+R7(K)
  313 CONTINUE
      TYPE*,' '
      R1(J)=R1(J)/K1
  310 CONTINUE
      DO 231 I=1,N
      P5(I)=R1(I)/CNT
      R1(I)=1-P5(I)
      R(I)=R1(I)
      SAVER(I)=R(I)
      ANUM=ANUM+R(I)
      DENOM=(N+1)*100.
      SAVEN(I)=N
      TYPE*,' '
  231 CONTINUE
      CNTRL=100.
      BIGC=ANUM/DENOM
  320 IF(IND.LT.1)GO TO 230
      DO 400 I=1,N
      SMALP(I)=R1(I)
      IF(ICYCL)350,390,390
  350 IF(SMALP(I).LE.0.) GO TO 330
      IF(SMALP(I).GE.1.) GO TO 340
      GO TO 380
  330 SMALP(I)=.0001
      GO TO 380
  340 SMALP(I)=.9999
  380 OBSY(I)=PROBIT(SMALP(I))
      GO TO 400
  390 OBSY(I)=EXPY(I)
  400 CONTINUE
C
C     COMPUTE STATISTICS
C
C
      SWN=0.
      SWNX=0.
      SWNY=0.
      SWNXX=0.
      SWNXY=0.
      SWNYY=0.
      SWNT=0.
      SWNXT=0.
      SWNTT=0.
      SWNTY=0.
      DO 470 I=1,N
      ED=OBSY(I)-5.
      IF(ABS(ED)-18.) 401,401,402
```

2.3.3 Listing of Computer Program for Probit Analysis of Algal Growth Test Data (Continued)

```
401 Z=.39894220401433*EXP(-(ED*ED*.5))
    GO TO 403
402 Z=.1D-15
403 IF (ICYCL) 410,420,420
410 BIGP (I)=SMALP(I)
    GO TO 440
420 AH=ABS(ED*SQRT(.5))
    IF(AH.LT.30.)  GO TO 430
    AI=.5
    GO TO 431
430 AI=((0.0000430638*AH+0.0002765672)*AH+0.0001520143)*AH+0.009
   12705272
    AI=.5-.5/(((AI*AH+.0422820123)*AH+.0705230784)*AH+1.)**16
431 BIGP(I)=.5D0+SIGN(AI,ED)
440 EXPY(I)=OBSY(I)+(SMALP(I)-BIGP(I))/Z
    WNP=100./(BIGP(I)+BIGC/(1.-BIGC))
    TP=(1.-BIGP(I))
    IF((BIGP(I).LT.1.).AND.(ABS(ED).LE.13.))  GO TO 441
    WN=0.
    GO TO 442
441 WN=WNP*Z*Z/TP
442 EX=X(I)
    EXWN=EX*WN
    PRBT=EXPY(I)
    SWN = SWN + WN
    YWN=PRBT*WN
    SWNYY = SWNYY + PRBT * YWN
    SWNX=SWNX+EXWN
    SWNY=SWNY+YWN
    SWNXX=SWNXX+EX*EXWN
    SWNXY=SWNXY+EX*YWN
    IF (ICYCL) 470,450,450
450 IF(SMALC)470,460,460
460 CONTINUE
    TWN=WNP*Z
    SWNT=SWNT+TWN
    SWNXT=SWNXT+EX*TWN
    SWNTT=SWNTT+WNP*TP
    SWNTY=SWNTY+TWN*PRBT
470 CONTINUE
    IF(SWN.EQ.0.) GO TO 520
    AVGX=SWNX/SWN
    AVGY=SWNY/SWN
    AVGT=SWNT/SWN
    SWNXX=SWNXX-SWNX*AVGX
    SWNXY=SWNXY-SWNX*AVGY
    SWNYY=SWNYY-SWNY*AVGY
    IF((SWNXX.EQ.0).OR.(SWNXY.EQ.0)) GO TO 520
    BNEW=SWNXY/SWNXX
    BB=BNEW
    A=AVGY-BNEW*AVGX
    IF(SMALC) 471,472,472
471 REGSS=BNEW*SWNXY
    C11=1./SWNXX
    C12=0.
    C22=0.
472 IF (ICYCL) 480,500,500
480 DO 490 I=1,N
    EXPY(I)=A+BNEW*X(I)
490 CONTINUE
    B=BNEW
```

```
      ICYCL=ICYCL+1
      GO TO 320
  500 IF(SMALC) 520,510,510
  510 SWNXT=SWNXT-SWNX*AVGT
      CNST=SMALC-BIGC
      SWNTY=SWNTY-SWNT*AVGY+((CNST/BIGC)*CNTRL)
      SWNTT=SWNTT-SWNT*AVGT+(1.-BIGC)/BIGC*CNTRL
      SWNYY=SWNYY+CNTRL*CNST/BIGC*CNST/(1.-BIGC)
      C11=1./(SWNXX-SWNXT/SWNTT*SWNXT)
      C12=1./(SWNXT-SWNTT/SWNXT*SWNXX)
      C22=1./(SWNTT-SWNXT/SWNXX*SWNXT)
      BNEW=C11*SWNXY+C12*SWNTY
  512 DOOMC=C12*SWNXY+C22*SWNTY
      A=AVGY-BNEW*AVGX-DOOMC*AVGT
      BIGC=BIGC+DOOMC*(1.-BIGC)
      REGSS=BNEW*SWNXY+DOOMC*SWNTY
C
C     COMPUTE EXPECTED PROBITS.
C     TEST ABS(B(I+1)-B(I)-.00001)
C     TEST ICYCL - 100
C
  520 DO 530 I=1,N
  530 EXPY(I)=A+BNEW*X(I)
      ICYCL=ICYCL+1
      TEST=BNEW-B
      XSQ=SWNYY-REGSS
      IF(TEST)540,550,550
  540 TEST=-TEST
  550 IF(ICYCL-100)560,580,580
  560 IF(SMALC)561,565,565
  561 IF(TEST.LE.00001)  GO TO 610
      GO TO 570
  565 IF((TEST.LE..00001).OR.(ABS(BNEW-BB).LT..00000005)) GO TO 610
  570 B=BNEW
      GO TO 320
  580 TEST=BNEW-B
  590 WRITE(5,600)TEST
  600 FORMAT(49H 100 CYCLES HAVE BEEN COMPLETED. B(100) - B(99) =,F14.7)
  610 L=0
      IF(K.EQ.0) GO TO 820
      IF(XSQ.GE.CHISQ(K))GO TO 650
      U=1.96
  645 WRITE(5,646)
  646 FORMAT(' CHI SQUARED NOT SIGNIFICANT.  EXPECTED NOS NOT CHECKED'/)
      GO TO 820
  660 FORMAT (51H NOTE; CHI SQUARED ESTIMATE OBTAINED BY SUBTRACTION)
  650 WRITE(5,660)
      U=T05(K)
  670 DO 680 I=1,N
C     SET UP EXPECTED NUMBERS.
  680 ENP(I)=100.*(BIGP(I)*(1.-BIGC)+BIGC)
  820 FK=K
      IF(K.EQ.0) GO TO 850
      HET=XSQ/FK
      FEXP=T05(K)*T05(K)
      FCAL=REGSS/HET
C     TEST REGRESSION SS.IF SIGNIFICANT
C     SET UP CONSTANTS AND COMPUTE STATISTICS.
      IF(FCAL-FEXP)830,830,850
  830 WRITE(5,840)
  840  FORMAT(  26H NONSIGNIFICANT REGRESSION/)
```

2.3.3 Listing of Computer Program for Probit Analysis of Algal Growth Test Data (Continued)

```
 850 G=U*U*C11/BNEW/BNEW
     DO 920 J=1,N
     EXPY(J)=BIGP(J)
     ENP(J)=N*EXPY(J)
     OBSY(J)=R1(J)
     IF(OBSY(J).LE.0.) GO TO 860
     IF(OBSY(J).GE.1.) GO TO 870
     OBSY(J)=PROBIT(OBSY(J))
     GO TO 920
 860 OBSY(J)=1.
     GO TO 920
 870 OBSY(J)=8.
 920 BIGP(J)=R1(J)-ENP(J)
     TYPE*,'------------------------------------------------------------
    1--------'
     TYPE*,' '
     TYPE*,' '
     TYPE*,'                 P R O B I T   T A B L E'
     WRITE(5,930)(X14(J),R1(J)*100.,EXPY(J),J=1,N)
 930 FORMAT(1X,13HCONCENTRATION,1X,9H PERCENT ,6X,11HPROBABILITY//(1X
    1,F8.4,6X,F6.2,6X,F10.4))
     SEB=SQRT(C11)
     IF(SMALC.LT.0)GO TO 932
     TEST=0.
     B=DEAD-TEST
 932 TYPE*,' '
     TYPE*,'------------------------------------------------------------------
    1----'
     WRITE(5,950)
 950 FORMAT(41HOTHE CONSTANTS USED IN THIS PROBLEM WERE;/)
     WRITE(5,960)HET
 960 FORMAT(6X,22HHETEROGENEITY FACTOR =,F9.4)
     WRITE(5,970)N
 970 FORMAT(10X,19HNUMBER OF POINTS = ,I3)
     WRITE(5,980)K
 980 FORMAT(8X,21HDEGREES OF FREEDOM = ,I3)
     WRITE(5,990)U
 990 FORMAT(19X,9HDEVIATE =,F9.4)
 940 WRITE(5,1010)ICYCL
1000 FORMAT(25X,6HG =    ,F9.4)
     TYPE*,' '
1010 FORMAT(8X,20H  NUMBER OF CYCLES =,I4,H.)
     WRITE(5,1020)
1020 FORMAT(22HOTHE STATISTICS ARE;    )
     WRITE(5,1030)AVGY
1030 FORMAT(21X,7HAVG Y =,F12.4)
     WRITE(5,1040)AVGX
1040 FORMAT(21X,7HAVG X = ,F12.4)
     WRITE(5,1050)AVGT
1050 FORMAT(21X,7HAVG T = ,F12.4)
     WRITE(5,1070)BNEW,SEB
1070 FORMAT(21X,7HSLOPE = ,F12.4,4X,4HSE =,F10.4)
     WRITE(5,1080)A
     WRITE(5,1000)G
1080 FORMAT(17X,11HINTERCEPT =,F12.4)
     WRITE(5,1090)XSQ
1090 FORMAT(15X,13HCHI SQUARED =,F12.4/)
     TYPE*,'_____________________________________________________________
    1_________________'
1105 WRITE(5,1100)
1100 FORMAT(5X,5HPOINT,6X,13HCONCENTRATION,12X,5HLOWER ,16X,5HUPPER)
```

2.3.3 Listing of Computer Program for Probit Analysis of Algal Growth Test Data (Continued)

```
C
      WRITE(5,987)
  987 FORMAT('                                                    95% CONFID
     1ENCE LIMITS')
      TYPE*,' '
C     COMPUTE FIDUCIAL LIMITS.
      OY=2.6737
      OP=0.01
      ONE=G/(1.-G)
      THREE=U/(BNEW*(1.-G))
      FIVE=G*(1./SWN+AVGT*AVGT*(C22-C12*C12/C11))
       J=1
      EM=(OY-A)/BNEW
      IF(THREE.LE.0.) GO TO 1150
 1110 EMLSX=EM-AVGX
      TWO=EMLSX-AVGT*C12/C11
      FOUR=1./SWN+AVGT*AVGT*C22-2.*EMLSX*AVGT*C12+EMLSX*EMLSX*C11
      PART2=EM+ONE*TWO
      PART3=THREE*SQRT(FOUR-FIVE)
      XLL=PART2-PART3
      XUL=PART2+PART3
      GO TO (1130,1120),ILOG
 1120 EM=EXP(2.302585*EM)
      XLL=EXP(2.302585*XLL)
      XUL=EXP(2.302585*XUL)
 1130 IF(EM.GT.X14(N))A999(J)=OP*100
      IF(EM.LT.X14(1))A998(J)=OP*100
      WRITE(5,1140) EM,XLL,XUL
 1140 FORMAT(3X,5H  EC1,3X,F16.4,5X,F16.4,3X,F16.4)
      GO TO 1190
 1150 GO TO (1170,1160),ILOG
 1160 EM=EXP(2.302585*EM)
 1170 WRITE(5,1180) EM
 1180 FORMAT(1X,5H  EC1,9X,F16.4,8X,'U N C A L C U L A B L E')
 1190 TYPE*,'-----------------------------------------------------------
     1-------------------'
      TYPE*,' '
      TYPE*,' '
      TYPE*,'NOTE: IF THE EC1  VALUE IS ABOVE/BELOW THE HIGHEST/LOWEST'
      TYPE*,'CONCENTRATION TESTED, EXTREME CAUTION SHOULD BE EXERCISED'
      TYPE*,'IN REGARD TO SUCH VALUE BECAUSE IT LIES OUTSIDE THE KNOWN'
      TYPE*,'RANGE AND IS ONLY AN EXTRAPOLATED ESTIMATED VALUE.SIMILAR'
      TYPE*,'CAUTIONS  SHOULD ALSO BE  USED IN REGARD TO THE UPPER AND'
      TYPE*,'LOWER 95% CONFIDENCE LIMITS FOR LIKE REASONS. SHOULD FUR-'
      TYPE*,'THER ASSISTANCE BE REQUIRED,PLEASE CONSULT YOUR QUALIFIED'
      TYPE*,'STATISTICIAN.'
      TYPE*,' '
      TYPE*,' '
      WRITE(5,9991)
 9991 FORMAT(///////////////////////////)
 9999 STOP
      END
      REAL FUNCTION PROBIT*8(PROP)
      IMPLICIT REAL*8 (A-H,O-Z)
      TEMP=DMIN1(PROP,1.-PROP)
      TEMP=SQRT(-2.*DLOG(TEMP))
      TEMP=TEMP-(2.515517+TEMP*(0.802853+TEMP*0.010328))/
     1  (1.+TEMP*(1.432788+TEMP*(0.189269+TEMP*0.001308)))
      PROBIT=5.+SIGN(TEMP,(PROP-0.5))
      RETURN
      END
```

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